UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA

Modulation of Human Apurinic/apyrimidinic Endonuclease 1 (APE1) Functions for Breast Cancer Therapy

Patrícia Isabel da Silva Guerreiro

Orientador: Professor Doutor Nuno Filipe Rocha Guerreiro de Oliveira

Co-orientadora: Professora Doutora Joana Paiva Gomes Miranda

Tese especialmente elaborada para a obtenção do grau de Doutor em Farmácia, especialidade de Toxicologia.

2016

UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA

Modulation of Human Apurinic/apyrimidinic Endonuclease 1 (APE1) Functions for Breast Cancer Therapy

Patrícia Isabel da Silva Guerreiro

Orientador: Professor Doutor Nuno Filipe Rocha Guerreiro de Oliveira

Co-orientadora: Professora Doutora Joana Paiva Gomes Miranda

Tese especialmente elaborada para a obtenção do grau de Doutor em Farmácia, especialidade de Toxicologia.

Júri

Presidente: Doutora Matilde da Luz dos Santos Duque da Fonseca e Castro, Professora Catedrática e Diretora da Faculdade de Farmácia da Universidade de Lisboa

Vogais: Doutora Maria de Lourdes Pinho de Almeida Souteiro Bastos, Professora Catedrática da Faculdade de Farmácia da Universidade do Porto

Doutor Fernando Manuel Gomes Remião, Professor Associado com Agregação da Faculdade de Farmácia da Universidade do Porto

Doutor António Sebastião Rodrigues, Professor Auxiliar da Faculdade de Ciências Médicas da Universidade Nova de Lisboa

Doutor Nuno Filipe Rocha Guerreiro de Oliveira, Professor Auxiliar com Agregação da Faculdade de Farmácia da Universidade de Lisboa, Orientador

Doutora Ana Paula Marreilha dos Santos, Professora Auxiliar da Faculdade de Farmácia da Universidade de Lisboa

Doutora Rita Alexandra do Nascimento Cardoso Guedes, Professora Auxiliar da Faculdade de Farmácia da Universidade de Lisboa

Trabalho financiado pela Fundação para a Ciência e a Tecnologia (FCT, Portugal) através da bolsa de doutoramento SFRH/BD/70293/2010 e do financiamento estratégico atribuído ao iMed.ULisboa (Pest-OE/SAU/UI4013/2011 e UID/DTP/04138/2013).

2016

To my parents, sister and Sérgio

Nothing in life is to be feared, it is only to be understood.

Now is the time to understand more, so that we may fear less.

Marie Curie | 1867-1934

Nobel Prize in Physics, 1903
Nobel Prize in Chemistry, 1911

ACKNOWLEDGEMENTS

First and foremost, I want to express my special gratitude to my supervisors.

To my supervisor Professor Nuno Oliveira my special thank for the guidance, tireless support, availability and critical thinking during numerous scientific discussions essential for the accomplishment of this thesis.

I also want to thank my co-supervisor Professor Joana Miranda for the support and helpful observations, especially in fundamental experimental issues and scientific writing.

A special word to Professor Matilde Castro for inviting me to apply to a doctoral grant and for introducing me to my supervisors. I also want to demonstrate my profound appreciation for your solicitude and availability despite your occupied schedule.

I also thank to Ana Sofia Fernandes for receiving me in the Chemical Biology and Toxicology group of the Research Institute for Medicines (iMed.ULisboa) and giving me the first insights in cell culture techniques. I appreciate the sharing of scientific knowledge as well as the collaboration in experimental work.

To Professor Fernando Antunes, a special thanks to the relentless support and availability regarding the biochemical activity assays.

To Sílvia Estácio and Professor Rita Guedes, thank you for showing me a piece of the emerging world of computational tools in drug discovery and for the important collaboration in the SBVS study based on docking analyses.

I also appreciate the collaboration of Nuno Saraiva in flow cytometry studies.

To all my former and current friends and colleagues of the Chemical Biology and Toxicology group, I would like to thank the friendship and bench sharing. A special thanks to Pedro Pinheiro and João Costa for the constant help and precious support. I also want to express my gratitude to Ana Matos for being a good listener and having always an encouraging word.

I also thank to my colleagues from Farmácia Holon Morais Soares.

To my dear friends Mara, Cláudia and Filipa, I am thankful for the support, friendship and sharing of important moments of your life.

To my family, especially to my parents, grandparents and little sister, I sincerely express my gratitude for the constant support, trust and unconditional love. I want to thank you for helping me to grow as a person and for believing in me. Importantly, I acknowledge your unmeasurable understanding for being less present, particularly in this last year. My little sister, my best friend, you have always something to say to encourage me.

To Sérgio, my bench colleague and my love, your attention, understanding, caring, encouragement and unconditional love were essential to accomplish this thesis. You appeared in my life in the hardest part of this work and, certainly, you are the best gift.

Finally, I want to acknowledge Faculty of Pharmacy, Universidade de Lisboa, and iMed.ULisboa for providing the infrastructures and conditions to perform this thesis. A special acknowledgement to Fundação para a Ciência e Tecnologia for funding this work through the PhD grant SFRH/BD/70293/2010 and strategic funding to iMed.ULisboa (Pest-OE/SAU/UI4013/2011 and UID/DTP/04138/2013).

ABSTRACT

DNA repair is required for the maintenance of genome stability. In the last years DNA repair pathways have emerged as important targets for cancer therapy. Since standard anticancer agents are mainly DNA-damaging drugs, its combination with DNA repair inhibitors may contribute to improve treatment outcomes. Among the multiple effectors involved in DNA repair, the multifunctional base excision repair (BER) protein apurinic/apyrimidinic endonuclease 1 (APE1) is one of the most attractive druggable targets in this field.

APE1 is the major endonuclease in BER participating in the repair of different DNA lesions including toxic abasic sites. In addition to the DNA repair activity, APE1 also acts independently as a reduction/oxidation signalling protein modulating the activation and DNA binding ability of several transcription factors implicated in cell survival and tumour promotion and progression. In this context, this thesis is focused on the combination of APE1 pharmacological inhibitors with conventional anticancer agents in the highly aggressive human breast cancer MDA-MB-231 cell line.

Endonuclease activity has been the most studied function of APE1 in cancer therapy. Methoxyamine (MX), a commercially available indirect inhibitor of APE1 DNA repair function, was evaluated in combination with doxorubicin (Dox) in MDA-MB-231 cells. The chemotherapeutic drug Dox is widely used in the treatment of advanced breast cancer and may act, in part, by inducing oxidative DNA damage. MX had little effects in viability and colony formation of MDA-MB-231 cells. However, a significant increase in the frequency of micronucleated cells and an alteration in the pattern of micronuclei distribution were observed suggesting an increase in Dox genotoxicity. The differential results obtained in terms of cytotoxicity and genotoxicity showed that a therapeutic strategy based on APE1 inhibition is likely to have no relevance for the improvement of outcomes of Dox treatment.

Although several putative inhibitors of APE1 endonuclease activity have been reported they still lack potential to be translated to the clinical setting. Therefore, in this thesis a structure-based virtual screening (SBVS) study based on molecular docking analysis of National Cancer Institute (NCI) database of compounds was performed to identify novel small-molecule inhibitors of APE1. The evaluation of SBVS study most promising compounds in a fluorescence-based APE1 endonuclease activity assay revealed three APE1 inhibitors. Compound 22 was a potent APE1 inhibitor showing inhibitory effects at nanomolar concentrations, while compounds 37 and 41 inhibited the enzyme in the

micromolar range. These novel scaffolds for the design of more potent APE1 inhibitors did not affect the viability of non-tumourigenic human breast epithelial MCF10A cell line highlighting their promising features.

The importance of APE1 modulation is beyond its functions in DNA repair. Therefore, E3330, a commercially available inhibitor of APE1 redox function, was also evaluated as single agent and in combination with the taxane drug docetaxel (DTX) in MDA-MB-231 cells. DTX has anti-migratory and anti-angiogenic effects and is frequently used in advanced breast cancer refractory to anthracycline-based regimens. Consequently, relevant endpoints of cell migration and invasion were studied in addition to cell viability, proliferation and cell cycle profile assessment. Minor effects were observed in cell proliferation. However, E3330 alone significantly reduced the collective cell migration evaluated by the wound-healing assay without affecting chemotaxis and chemoinvasion. The combination of E3330 with DTX significantly decreased invasion of MDA-MB-231 cells suggesting a potential therapeutic role in metastatic breast cancer.

The results described in this work emphasise the importance of preclinical studies of APE1 functions in cancer therapy and highlight the potential of novel drug combinations based on APE1 inhibitors reinforcing the role of targeting DNA repair in cancer treatment.

KEYWORDS

Apurinic/apyrimidinic endonuclease 1 (APE1), breast cancer therapy, DNA repair, novel APE1 inhibitors, redox activity.

RESUMO

As células de mamífero desenvolveram um conjunto de mecanismos que atuam de forma regulada e coordenada para proteger a integridade do genoma. Estes mecanismos incluem as vias de reparação de DNA responsáveis pela reparação de lesões resultantes da exposição constante do material genético a agentes químicos e físicos exógenos, assim como a produtos endógenos resultantes dos processos celulares como é o caso das espécies reativas de oxigénio (ROS). As várias vias de reparação de DNA podem atuar de forma coordenada ou independente de acordo com a complexidade das lesões.

Nos últimos anos, as proteínas das vias de reparação de DNA têm sido consideradas importantes alvos terapêuticos no tratamento do cancro. A atual terapêutica do cancro baseia-se na utilização de agentes indutores de lesões de DNA com o objetivo de induzir a acumulação de lesões citotóxicas que ultrapassem a capacidade de reparação das células tumorais culminando na morte celular. Para além disso, as células tumorais apresentam frequentemente mutações em genes que codificam proteínas envolvidas na reparação. Desta forma, a inibição de uma via de reparação, responsável por compensar a incapacidade de reparação do DNA da via que apresenta as mutações, poderá ser uma estratégia terapêutica que aumente a eficácia e diminua a toxicidade dos agentes antitumorais. Por outro lado, existe uma redundância de funções nos mecanismos de reparação de DNA que pode contribuir para a resistência aos fármacos antitumorais. Neste contexto, a modulação das vias de reparação de DNA constitui uma importante estratégia terapêutica no tratamento do cancro.

A via de reparação por excisão de bases (BER) é a principal responsável pela remoção e reparação de pequenas lesões induzidas por oxidação, alquilação e desaminação de bases do DNA. A BER também reconhece e remove o uracilo presente no DNA e está envolvida na reparação de locais abásicos citotóxicos espontaneamente induzidos durante o metabolismo celular ou formados como produtos intermédios durante a reparação de bases lesadas. A BER participa ainda na reparação de quebras de cadeias simples (SSBs).

De acordo com o tipo de lesão e o tamanho do fragmento de nucleótidos a remover após a excisão da base por DNA glicosilases, a reparação pode ocorrer através de duas sub-vias da BER designadas por *short-patch* (SP-BER) e *long-patch* (LP-BER).

A enzima endonuclease apurínica/apirimidínica 1 (APE1) é a principal endonuclease nas células de mamíferos responsável pela incisão do local abásico resultante da remoção das lesões não volumosas do DNA. Esta enzima multifuncional participa nas duas sub-vias da BER, apresentando-se como um importante alvo terapêutico para aumentar a sensibilidade a diferentes agentes antitumorais como demonstrado por vários estudos pré-clínicos e clínicos. Para além disso, alterações de expressão e da distribuição intracelular da APE1 têm sido correlacionadas com o prognóstico de diversos tipos de cancro, nomeadamente o cancro da mama.

Apesar de ser uma importante enzima de reparação, a APE1 também tem um papel independente como proteína de redução/oxidação em vias de sinalização celular, modulando a ativação e a capacidade de ligação ao DNA de vários fatores de transcrição envolvidos na sobrevivência celular e na progressão tumoral. A modulação da função redox da APE1 constitui também uma estratégia terapêutica relevante uma vez que está envolvida na regulação simultânea de vários processos necessários à metastização.

Neste âmbito, a presente tese visa a avaliação de uma estratégia terapêutica que combina inibidores da atividade endonucleásica e/ou da função redox da APE1, comercialmente disponíveis, com fármacos antitumorais atualmente utilizados na terapêutica do cancro da mama. Para além disso, perante a relevância da função de reparação de DNA da APE1 e a inexistência de inibidores da APE1 com potencial para serem utilizados na clínica, este trabalho pretende também identificar novos inibidores da atividade endonucleásica desta enzima.

Os tumores da mama constituem o tipo de cancro mais comum nas mulheres. A elevada mortalidade do cancro da mama deve-se, principalmente, ao desenvolvimento de metástases. Uma vez que a APE1 tem sido associada a prognósticos menos favoráveis no cancro da mama, a linha celular MDA-MB-231, representativa de adenocarcinoma da mama agressivo, com capacidades invasivas *in vitro* e tumorigénicas *in vivo* foi selecionada como modelo de cancro da mama em estadio avançado neste trabalho.

A antraciclina doxorrubicina (Dox) é um dos fármacos antitumorais mais utilizados no tratamento do cancro da mama em estadio avançado. Apesar da complexidade dos mecanismos de ação da Dox não se encontrar completamente elucidada, este fármaco pode induzir a formação de ROS que por sua vez podem provocar a oxidação de bases de DNA. A Dox também é um fármaco com propriedades genotóxicas reconhecidas, nomeadamente com características aneugénicas e clastogénicas, que

também pode induzir SSBs. Ambos os tipos de lesões podem ser alvos de reparação pela BER. Desta forma, a metoxiamina (MX), um inibidor indireto da função da APE1 comercialmente disponível e em estudos clínicos de fase I, foi estudada em combinação com a Dox em células MDA-MB-231. Várias concentrações dos compostos e diferentes protocolos de exposição foram adotados para mimetizar a administração dos fármacos na clínica. Diferentes métodos foram também utilizados para avaliar os efeitos da combinação do fármaco citotóxico Dox com a MX em termos de viabilidade celular (ensaio do MTT e do violeta de cristal), proliferação celular (ensaio de formação de colónias) e genotoxicidade. Enquanto que a utilização da MX apresentou um ligeiro efeito sensibilizador da viabilidade e proliferação das células tratadas com Dox, um efeito significativo foi observado na genotoxicidade. O ensaio do micronúcleo em células com a citocinese bloqueada (ensaio CBMN) revelou um aumento pronunciado na frequência das células micronucleadas e uma alteração no padrão de distribuição dos micronúcleos sugerindo um aumento da genotoxicidade da Dox na presença da MX. Contudo, estes resultados mostram que as células MDA-MB-231 têm uma capacidade para tolerar os efeitos genotóxicos da combinação do inibidor da APE1 com a Dox evidenciando uma ausência de relevância desta estratégia terapêutica para melhorar os resultados do tratamento do cancro da mama com Dox. Estes resultados podem estar associados aos diferentes mecanismos de ação descritos para a Dox e à redundância das vias de reparação.

Apesar de, atualmente, vários possíveis inibidores da actividade endonucleásica da APE1 se encontrarem descritos na literatura, estes não têm evidenciado potencial para a sua utilização na clínica. Desta forma, neste trabalho também foi proposta a identificação de novos inibidores diretos da APE1. Um estudo computacional de *screening* virtual baseado na estrutura (SBVS) e em análises de *docking* molecular foi realizado com os compostos químicos depositados no repositório do National Cancer Institute (NCI). Os compostos mais promissores identificados no estudo computacional foram avaliados num ensaio bioquímico de atividade endonucleásica da APE1 baseado na fluorescência para identificar potenciais inibidores desta enzima. Os compostos 22, 37 e 41 mostraram a capacidade para inibir a APE1. De facto, o composto 22 demonstrou ser um dos mais potentes inibidores da APE1 identificados até à data, inibindo a enzima quando utilizado em concentrações na ordem dos nanomolar. Por sua vez, concentrações na ordem dos micromolar dos compostos 37 e 41 inibiram a APE1. A inibição da APE1 foi também avaliada num ensaio mecanisticamente diferente para confirmar o carácter inibidor destes compostos. É de notar que os compostos 22, 37 e 41 não apresentaram efeitos na viabilidade celular

das células de mama não tumorais MCF10A. Para além disso, a presença de grupos sulfonato e isotiocianato no composto 22 evidencia a relevância destes grupos funcionais para o desenvolvimento de novos inibidores potentes da APE1.

A importância da modulação da APE1 não se deve apenas à sua função na reparação do DNA mas também à sua atividade como factor de sinalização redox. Neste contexto, devido ao papel emergente das espécies reativas e da regulação redox no desenvolvimento de agentes antitumorais, o inibidor redox comercialmente disponível da APE1, o E3330 foi estudado individualmente e em combinação com o taxano docetaxel (DTX) em células MDA-MB-231. O DTX é um fármaco antitumoral frequentemente utilizado no cancro da mama avançado refratário ao tratamento com antraciclinas. O seu mecanismo de ação baseia-se na indução de uma hiperestabilização dos microtúbulos. Para além disso, o DTX apresenta propriedades antimigratórias e antiangiogénicas. Consequentemente, para além dos efeitos na viabilidade, proliferação e ciclo celular, também foram avaliados os efeitos na migração e invasão celular. Enquanto que os efeitos observados na proliferação celular foram ligeiros, o E3330 diminuiu significativamente a migração celular coletiva sem afetar a quimiotaxia e a quimioinvasão. Por sua vez, a combinação do E3330 com o DTX reduziu significativamente a invasão das células MDA-MB-231 sugerindo um potencial terapêutico no cancro da mama metastático. É de notar, que até à data, este parece ser o primeiro estudo focado na avaliação da combinação terapêutica destes dois compostos.

De um modo geral, os resultados descritos nesta tese realçam a importância de se efetuarem estudos pré-clínicos sobre as diferentes funções da APE1 na terapêutica do cancro e evidenciam o potencial de novas combinações de fármacos e do desenvolvimento de novos inibidores da APE1, reforçando o seu papel no tratamento do cancro.

PALAVRAS-CHAVE

Endonuclease apurínica/apirimidínica 1 (APE1), terapia do cancro da mama, reparação de DNA, novos inibidores da APE1, atividade redox

LIST OF PUBLICATIONS AND COMMUNICATIONS

From the results presented in this thesis, the following full papers were published in international peer-reviewed journals:

1. **Guerreiro PS**, Corvacho E, Costa JG, Saraiva N, Fernandes AS, Castro M, Miranda JP, Oliveira NG. The APE1 redox inhibitor E3330 reduces collective cell migration of human breast cancer cells decreases chemoinvasion and colony formation when combined with docetaxel. Chem Biol Drug Des. *(submitted)*.

2. **Guerreiro PS**, Estácio SG, Antunes F, Fernandes AS, Pinheiro PF, Costa JG, Castro M, Miranda JP, Guedes RC, Oliveira NG. Structure-based virtual screening toward the discovery of novel inhibitors of the DNA repair activity of the human apurinic/apyrimidinic endonuclease 1. Chem. Biol. Drug Des. 2016; 88:915–925.

3. **Guerreiro PS**, Fernandes AS, Costa JG, Castro M, Miranda JP, Oliveira NG. Differential effects of methoxyamine on doxorubicin cytotoxicity and genotoxicity in MDA-MB-231 human breast cancer cells. Mutat Res. 2013; 757:140–147.

Under the scope of this thesis, the following book chapter was also published:

1. Rodrigues AS, Gomes BC, Martins C, Gromicho M, Oliveira NG, **Guerreiro PS**, *et al.*. DNA repair and resistance to cancer therapy, in: C. Chen (Ed.), New Research Directions in DNA repair, InTech, Rijeka, 2013, pp. 489–528.

The work herein presented contains methods described in the following full-length papers published during this thesis:

1. Costa JC, Saraiva N, **Guerreiro PS**, Louro H, Silva MJ, Miranda JP, Castro M, Batinić-Haberle I, Fernandes AS, Oliveira NG. Ochratoxin A-induced cytotoxicity, genotoxicity and reactive oxygen species in kidney cell: an integrative approach of complementary endpoints. Food Chem Toxicol. 2016; 87:65–76.

2. Ribeiro IAC, Faustino CMC, **Guerreiro PS**, Frade RFM, Bronze MR, Castro MF, Ribeiro MHL. Development of novel sophorolipids with improved cytotoxic activity toward MDA-MB-231 breast cancer cells. J Mol Recognit. 2015; 28:155–165.

3. Bandarra S, Fernandes AS, Magro I, **Guerreiro PS**, Pingarilho M, Churchell MI, Gil OM, Batinić-Haberle I, Gonçalves S, Rueff J, Miranda JP, Marques MM, Beland FA, Castro M, Gaspar JF, Oliveira NG. Mechanistic insights into the cytotoxicity and genotoxicity induced by glycidamide in human mammary cells. Mutagenesis. 2013; 28:721–729.

4. Gonçalves S, Fernandes AS, Oliveira NG, Marques J, Costa J, Cabral MF, Miranda J, **Guerreiro PS**, Castro M. Cytotoxic effects of cadmium in mammary epithelial cells: protective role of the macrocycle [15]pyN5. Food Chem Toxicol. 2012; 50:2180–2187.

During this thesis, the following oral communications were presented in scientific meetings:

1. **Guerreiro PS**, Corvacho E, Miranda JP, Costa JG, Fernandes AS, Castro M, Oliveira NG. Inhibition of APE1 redox function with E3330: effects on the proliferation and migration of MDA-MB-231 breast cancer cells. XLV Reunião Anual da Sociedade Portuguesa de Farmacologia, 2015, Lisbon, Portugal.

2. **Guerreiro PS**, Estácio S, Miranda JP, Antunes F, Costa JG, Fernandes AS, Castro M, Guedes R, Oliveira NG. APE1 DNA repair activity: a druggable target for the development of chemotherapeutic sensitizers. 5th Post-Graduate iMed.UL Students Meeting, 2013, Lisbon, Portugal.

3. **Guerreiro PS**, Estácio S, Miranda JP, Antunes F, Fernandes AS, Castro M, Guedes R, Oliveira NG. Development of APE1 inhibitors as potential sensitizing agents for chemotherapeutic drugs. XLIII Reunião Anual da Sociedade Portuguesa de Farmacologia, 2013, Porto, Portugal.

The following poster communications were also presented in scientific meetings:

1. **Guerreiro PS**, Corvacho E, Miranda JP, Costa JG, Fernandes AS, Castro M, Oliveira NG. Targeting APE1 redox function with E3330: effects on the migration of MDA-MB-231 cells. 51st Congress of the European Societies of Toxicology (Eurotox 2015), 2015, Porto, Portugal.

2. **Guerreiro PS**, Corvacho E, Costa JG, Fernandes AS, Castro M, Saraiva N, Miranda JP, Oliveira NG. Evaluation of an inhibitor of APE1 redox function in docetaxel-treated MDA-MB-231 cells. 7th iMed.ULisboa Postgraduate Students Meeting, 2015, Lisbon, Portugal.

3. **Guerreiro PS**, Corvacho E, Miranda JP, Costa JG, Fernandes AS, Castro M, Oliveira NG. Targeting APE1 redox activity to modulate the migration of MDA-MB-231 breast cancer cells. 6th iMed.ULisboa Postgraduate Students Meeting, 2014, Lisbon, Portugal.

4. **Guerreiro PS**, Miranda JP, Estácio S, Antunes F, Costa JG, Fernandes AS, Castro M, Guedes R, Oliveira NG. Small-molecule inhibitors of APE1 DNA repair activity as potential sensitizers of chemotherapy. The DNA damage response in cell physiology and disease - EMBO Conference, 2013, Cape Sounio, Grécia.

5. **Guerreiro PS**, Miranda JP, Fernandes AS, Costa JG, Castro M, Oliveira NG. Effect of APE1 inhibitors on the cytotoxicity and genotoxicity of doxorubicin in MDA-MB-231 cells. 49th Congress of the European Societies of Toxicology (Eurotox 2013), 2013, Interlaken, Switzerland.

6. **Guerreiro PS**, Estácio S, Miranda JP, Fernandes AS, Castro M, Guedes R, Oliveira NG. APE1 inhibitors as potential sensitizing agents for chemotherapeutic drugs. 4th Post-Graduate iMed.UL Students Meeting, 2012, Lisbon, Portugal.

7. **Guerreiro P**, Fernandes AS, Miranda JP, Castro M, Oliveira NG. Effect of the indirect APE1 inhibitor methoxyamine on the cytotoxicity induced by doxorubicin in metastatic breast cancer cells. 3rd Post-Graduate iMed.UL Students Meeting, 2011, Lisbon, Portugal.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

3-AB	3-aminobenzamide
3-D	Three-dimensional
4-AN	4-amino-1,8-naphthalimide
AAG/MPG	N-methylpurine-DNA glycosylase
AP	Apurinic/apyrimidinic
AP-1	Activator protein 1
APE1	Human apurinic/apyrimidinic endonuclease 1
ATP	Adenosine triphosphate
B-Raf	B-type Raf kinase
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BER	Base excision repair
BHQ-2	Black hole quencher-2
BN	Binucleated
BRCA1/2	Breast cancer 1/2, early onset gene
CREB	cAMP response element-binding protein
CRT0044876	7-nitro-1*H*-indole-2-carboxylic acid
CV	Crystal violet
DDR	DNA damage response
DHE	Dihydroethidium
Dox	Doxorubicin
dRP	5'-deoxyribose phosphate
DSB	Double-strand break
DMSO	Dimethylsulfoxide
E3330	(2E)-2-[(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)methylene] undecanoic acid
Egr-1	Early growth response protein 1
EMSA	Electrophoretic mobility shift assay

5-FU	5-Fluorouracil
FA	Fanconi anemia
FapyG	2,6-diamino-4-hydroxy-5-formamidopyrimidine
FEN1	Flap endonuclease 1
GA	Genetic algorithm
GG-NER	Global-genome nucleotide excision repair
GOLD 5.1.0	Genetic Optimization for Ligand Docking
H_2O_2	Hydrogen peroxide
HER2/neu	Human epidermal growth factor receptor 2
HIF-α	Hypoxia-inducible factor 1 alpha
^{1}H NMR	Proton nuclear magnetic resonance
HPLC-DAD	High-performance liquid chromatography with a diode array detector
HR	Homologous recombination
HR-ESI-MS	High-resolution electrospray ionization mass spectrometry
Hsp70	Heat shock protein 70
HTS	High-throughput screening
ICL	Interstrand cross-link
IdUrd	5-iodo-2'-deoxyuridine
IR	Ionising radiation
LP-BER	Long-patch BER
MBC	Metastatic breast cancer
MBD4	Methyl-CpG binding domain 4 DNA glycosylase
MDM2	Mouse double minute 2
MDR1	Multidrug resistance gene
MMP	Matrix metalloproteinase
MMR	Mismatch repair
MMS	Methyl methanesulfonate
MN	Micronucleus

MNBN	Micronucleated binucleated
MOE	Molecular Operating Environment
mRNA	Messenger RNA
MTS	Mitochondrial targeting sequence
MTT	Thiazolyl blue tetrazolium bromide
MUTYH	MutY homolog DNA glycosylase
MX	Methoxyamine hydrochloride
nCaRE	Negative calcium responsive elements
NCI/DTP	National Cancer Institute/Developmental Therapeutics Program
NDI	Nuclear division index
NEIL1/2/3	NEI endonuclease VIII-like 1/2/3
NER	Nucleotide excision repair
NF-κB	Nuclear factor-kappa B
NHEJ	Non-homologous end-joining
NIR	Nucleotide incision repair
NSCLC	Non-small cell lung cancer
NTHL1	*E. coli* NTH endonuclease III-like DNA glycosylase
NLS	Nuclear localization sequence
NPM1	Nucleophosmin 1 protein
$O_2^{\bullet-}$	Superoxide anion
OGG1	8-Oxoguanine-DNA glycosylase
5'-P	5'-Phosphate
3'-PG	3'-Phosphoglycolate
3'-PUA	3'-Phospho-α,β-unsaturated aldehyde
PARP	Poly(ADP-ribose)polymerase
PCNA	Proliferating cell nuclear antigen
PDB	Protein Data Bank
PNKP	Polynucleotide kinase/phosphatase
Polβ/δ/ε	DNA polymerase β/δ/ε

PTEN	Phosphatase and tensin homolog
PTH	Parathyroid hormone
QSAR	Quantitative structure-activity relationship
Ref-1	Redox effector factor 1
RFC	Replication factor C
RMSD	Root mean square deviation
RNase H	Ribonuclease H
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SBVS	Structure-based virtual screening
SCCHN	Squamous cell carcinoma of the head and neck
shRNA	Short-hairpin RNA
siRNA	Small-interfering RNA
SMUG1	Single-strand-selective monofunctional uracil-DNA glycosylase 1
SP-BER	Short-patch BER
SSB	Single-strand break
STAT3	Signal transducer and activator of transcription 3
TAMRA	Carboxytetramethylrhodamine
TBHP	*Tert*-butylhydroperoxide
TC-NER	Transcription-coupled nucleotide excision repair
TDG	Thymine-DNA glycosylase
THF	Tetrahydrofuran
TLS	Translesion synthesis
TMZ	Temozolomide
TNBC	Triple-negative breast cancer
UNG	Uracil-DNA glycosylase
XRCC1	X-ray cross-complementing factor 1
YB-1	Y-box-binding protein 1

CHAPTER 1

GENERAL INTRODUCTION

This Chapter contains information published in:

Rodrigues AS, Gomes BC, Martins C, Gromicho M, Oliveira NG, Guerreiro PS, et al.. DNA repair and resistance to cancer therapy, in: C. Chen (Ed.), New Research Directions in DNA repair, InTech, Rijeka, 2013, pp. 489-528.

1.1. DNA REPAIR PATHWAYS AND CANCER THERAPY – A BRIEF OVERVIEW

DNA is continuously exposed to numerous sources of damage which include exogenous chemical and physical agents and endogenous genotoxic insults related to physiological and cellular processes namely the reactive species generated by metabolism and the replication errors [1,2]. Cells have evolved a set of tightly regulated, coordinated and redundant surveillance mechanisms to protect the genome stability. These complex molecular pathways are collectively named DNA damage response (DDR) and they detect, signal, control cell cycle progression, promote the DNA repair and activate cell death machineries to counteract the thousands of lesions that threat the genome integrity (Fig. 1.1) [1–6].

In general, a DNA lesion is recognized by several sensor proteins of DDR. These DNA damage sensors initiate signalling pathways that can slow down or transiently arrest the cell cycle progression increasing the available time for DNA repair [2,4]. Cells have multiple DNA repair pathways to prevent the accumulation of a wide diversity of DNA lesions. These DNA repair mechanisms target different types of DNA damage and they can act independently or interact according to the complexity of DNA lesions [1,2].

The major DNA repair pathways include the a) direct reversal repair, b) mismatch repair (MMR) c) base excision repair (BER), d) nucleotide excision repair (NER), e) translesion synthesis (TLS), f) homologous recombination (HR), g) non-homologous end-joining (NHEJ) and h) the Fanconi anemia (FA) pathways [1,2]. The present work is focused on human apurinic/apyrimidinic endonuclease 1 (APE1), a key BER enzyme considered a promising target for cancer therapy, including breast cancer. In this context, a detailed description of the other mentioned DNA repair pathways is beyond the scope of this thesis. Several comprehensive reviews on this topic have been published in the last years [1,2,7–13].

Although the majority of the DNA repair systems require the coordinated action of several proteins, a small group of DNA lesions can be directly repaired by a single protein mechanism without the incision of the DNA backbone or the base excision. The direct reversal repair is a simple and error-free DNA repair process with high substrate specificity [14,15]. In addition, the BER pathway identifies and excises bases damaged by alkylation, oxidation, deamination, depurination/depyrimidination, removes the uracil from DNA and also repairs DNA single-strand breaks (SSBs) [2,16]. While BER repairs small adducts, NER ensures the removal of bulky helix-distorting base lesions which may affect the DNA replication and transcription. In NER, a single-stranded DNA

segment containing the damaged base, usually with 24–32 oligonucleotides of length, has to be excised and replaced to complete the repair. NER may proceed through two sub-pathways depending on the substrate: the global-genome NER (GG-NER) targets lesions in the entire genome and the transcription-coupled NER (TC-NER) is involved in the removal of transcription-blocking damage to allow an accurate gene expression [13,16,17]. The MMR eliminates mispaired bases and small insertion-deletion loops generated during DNA replication [2,16,18]. Moreover, mammalian cells use the TLS to

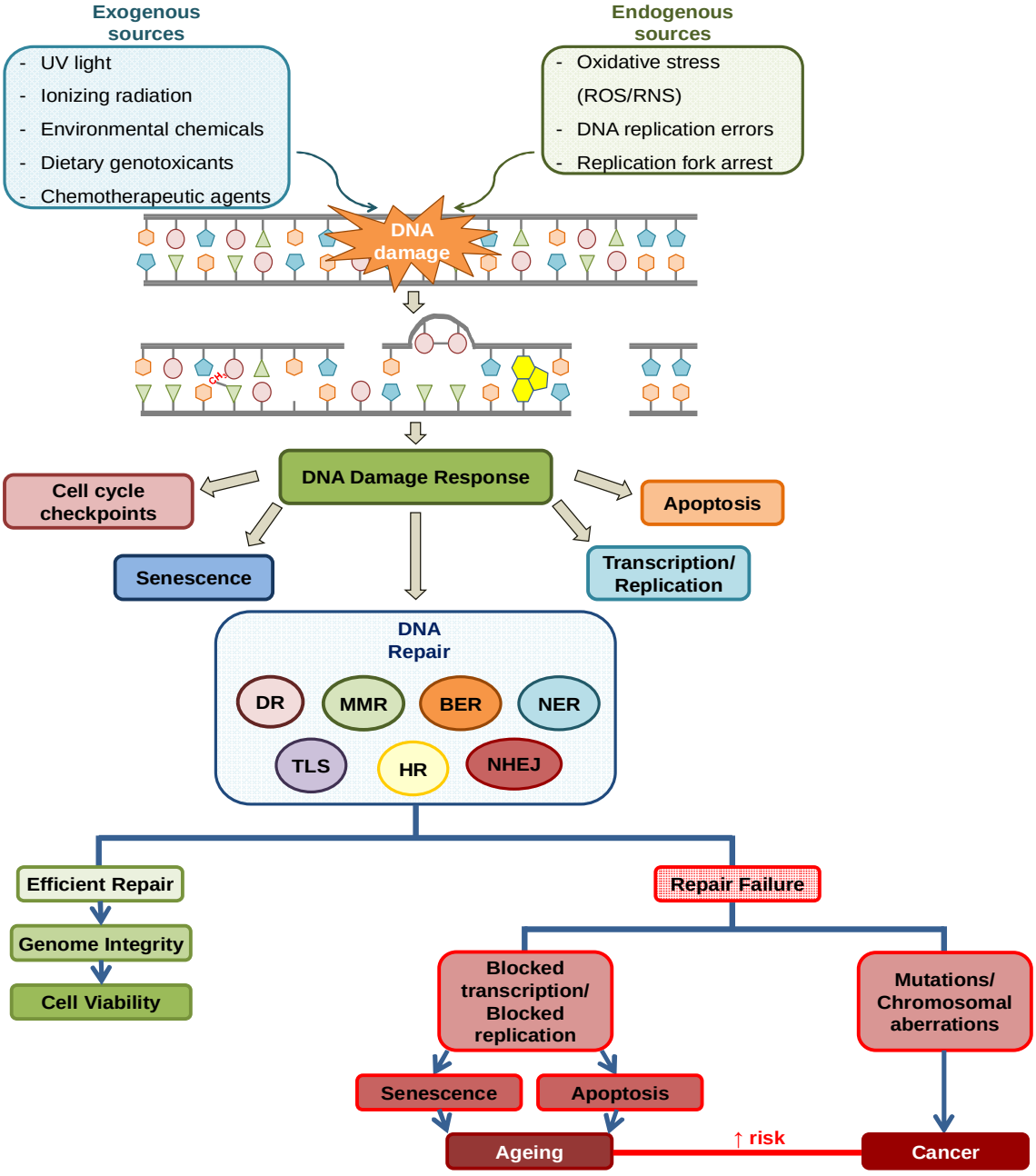


Figure 1.1 Sources of DNA damage and general overview of DNA repair pathways and biological responses to DNA lesions [2,3,6]. *Abbreviations: ROS - reactive oxygen species; RNS - reactive nitrogen species; DR - direct reversal repair; MMR - mismatch repair; BER - base excision repair; NER - nucleotide excision repair; TLS - translesion synthesis; HR - homologous recombination; NHEJ - non-homologous end-joining.*

bypass the lesions that could be incompletely repaired and often remain in the DNA during replication jeopardizing the progress and the fidelity of replicative polymerases [8,19].

For DNA double-strand breaks (DSBs), two major DNA repair pathways are available: the NHEJ and the HR. NHEJ repairs the DSBs by promoting the direct ligation of broken DNA ends. Conversely, HR requires an homologous undamaged DNA template to carry out the repair of DSBs, interstrand cross-links (ICLs) and to restart stalled replication forks. Thus, NHEJ is considered an error-prone mechanism which can be activated in all phases of the cell cycle whereas HR is generally classified as an error-free repair strategy primarily restricted to late S and G2 phase. In fact, HR is more prominent after the DNA replication because an intact sister chromatid can be used as template for repair [2,16,20,21]. For ICLs repair, which cannot be completely removed by MMR and/or NER, HR cooperates with the FA pathway. In general, the repair of these lesions can occur only in the S-phase during the DNA replication [2,22].

The outcomes of DNA lesions are unpredictable despite cell efforts for the genome maintenance. They depend not only on the nature of the damage but also on the number and location of DNA lesions, cell type, cell cycle phase and differentiation stage [6]. An inaccurate repair and/or the overwhelming of the DNA repair mechanisms yield the accumulation of DNA lesions (Fig. 1.1). The blockage of transcription or replication is a common consequence inducing senescence or cell death, particularly in non-proliferating cells, and contributing for ageing [6,23]. On the other hand, in proliferating cells permanent mutations appear during the replication cycles of damaged DNA. Similarly, structural chromosomal aberrations may be generated during the DSBs repair and dysregulate gene expression. The transmission of these alterations to descendant cells can also result in mutations. The inactivation of certain tumour suppressor genes, the activation of oncogenes and an enhanced genomic instability are potential effects of these harmful mutations. Consequently, if these alterations constitute a selective advantage for cells they may contribute to an uncontrolled and sustained cellular proliferation leading to the development of precancerous lesions and ultimately to cancer progression [2,6,23,24]. Mutations can also affect genes required for DNA repair such as breast cancer 1/2, early onset genes (*BRCA1* and *BRCA2)*. Both are tumour suppressor genes which encode proteins involved in DDR and HR and inherited germline mutations in *BRCA1* and *BRCA2* have been correlated with a higher susceptibility to develop hereditary breast and ovarian cancer among others [25–27]. The epigenetic alterations which involve the chromatin remodelling, histones modification and the DNA methylation also display a

carcinogenic role through the inactivation of the DDR and DNA repair effectors and enabling the malignant transformation of cells and tumour development [2,6,23,24].

Another important aspect regarding DNA repair and cancer is the resistance to anticancer agents. Cancer therapy is challenging and generally it involves the use of different strategies to target specific molecular and cellular features of cancer cells in order to cure, or at least hamper the tumour progression.

The most fundamental hallmark of cancer cells is their ability to proliferate at higher rates than non-tumour cells which renders the cell cycle an attractive target in cancer therapy [28]. In addition to the inhibitors of the mitotic spindle and the targeting of growth signalling pathways, cell cycle is frequently disrupted by the DNA-damaging agents. Actually, many chemotherapeutic regimens are based on DNA-damaging drugs often used in complementary combinations [2,8]. Their effectiveness depends on their ability to directly or indirectly damage DNA with the generation of cytotoxic lesions that overwhelm the DNA repair capacity and ultimately induce cell death of rapid proliferating cancer cells (Table 1.1). In view of this, the rationale for their use in cancer therapy is, as abovementioned, the genomic instability and replicative stress of tumour cells as well as the presence of mutator phenotypes in DNA repair genes which can impair or even inactivate DNA repair pathways increasing the cell death induced by DNA-damaging drugs [2,8,29].

However, the efficacy of DNA-damaging drugs is frequently precluded by toxicity issues and cancer resistance to therapy. Although non-tumour cells have a lower replication rate and are usually proficient in the DDR being less susceptible to the effects of ionising radiation (IR) and chemotherapeutic drugs, their DNA can also be damaged with the subsequent development of side effects [8]. In respect to the resistance to DNA-damage agents, there is a redundancy of DNA repair mechanisms that may lead to the activation of another DNA repair pathway upon failure of the first attempt to repair the lesions. Moreover, cancer cells may also present an increased activity of the DNA-damage signalling and DNA repair pathways preventing the accumulation of toxic lesions and promoting their survival [2,8,16,30,31].

Table 1.1 Anticancer agents and DNA repair pathways involved in the repair of DNA damage-induced by cancer treatment. The major DNA repair pathways contributing to the repair of lesions are represented in bold. Adapted from [2,8,29,32].

Anticancer agents	Types of DNA lesions	DNA repair pathways
Radiotherapy and radiomimetics		
Ionizing radiation; Bleomycin	DSBs; SSBs; Base damage	**NHEJ**; **BER**; HR
Monofunctional alkylators		
Alkylsulphonates (e.g. busulfan); Nitrosoureas (e.g. BCNU); Temozolomide (TMZ)	Base damage; Replication lesions; Bulky DNA adducts	DR; **BER**; MMR; **HR**; NER; TLS; FA
Bifunctional alkylators		
Nitrogen mustards (e.g. cyclophosphamide); Mytomicin C; Cisplatin	DSBs; DNA crosslinks; Replication lesions; Bulky DNA adducts	**NER**; **HR**; FA; TLS; MMR
Antimetabolites		
5-Fluorouracil (5-FU); Thiopurines (e.g. 6-mercaptopurine); Folate analogues (e.g. methotrexate); Gemcitabine/Troxacitabine	Base damage; Replication lesions	**BER**; MMR
Topoisomerase inhibitors		
Camptothecin (Topo I); Etoposide (Topo II); Anthracyclines (Topo II)	DSBs; SSBs; Replication lesions; Oxidative base damage	**HR**; NHEJ; **FA**; BER
Taxanes		
Docetaxel; Paclitaxel	Taxanes-induced ROS [32,33] -oxidative base damage (?)	BER (?)
Replication inhibitors		
Hydroxyurea; Aphidicolin	DSBs; Replication lesions	**HR**; **FA**; NHEJ

In this context, the modulation of DNA repair is a targeted therapeutic approach to improve the outcomes of cancer treatments (Fig. 1.2). DNA repair inhibitors may not only overcome the resistance to anticancer agents but also increase their sensitivity and specificity decreasing the toxic side effects. This is particularly important in tumours with somatic mutations that lead to the inactivation of a certain DNA repair pathway. In these circumstances cancer cells will be more dependent on the remaining

DNA repair pathways which could be targeted with DNA repair inhibitors to induce a hypersensitivity to anticancer drugs and decrease the toxic side effects [2,8,16]. The concept of synthetic lethality has also emerged as a therapeutic approach in tumours carrying genetic defects [8,29,33]. The underlying principle of synthetic lethality is the cells ability to tolerate and maintain viability upon inactivation of only one pathway due to mutations of genes that code relevant proteins [8,29,33]. The use of chemical inhibitors to abrogate a second and redundant DNA repair pathway promotes the accumulation of DNA damage and induces cell death [8,29,33]. Synthetic lethality has been notably relevant in tumour cells harbouring mutations in proteins associated with defects in HR which have shown to be sensitive to inhibitors of proteins involved in BER, namely poly(ADP-ribose)polymerase 1 (PARP1) [29,34,35]. These are clinical opportunities to personalised therapy since it requires the knowledge of tumour genetic alterations to improve the specificity of the treatment, the overall survival and to the use of DNA repair inhibitors in monotherapy limiting the number of drugs administered and consequently the toxicity of therapeutic agents.

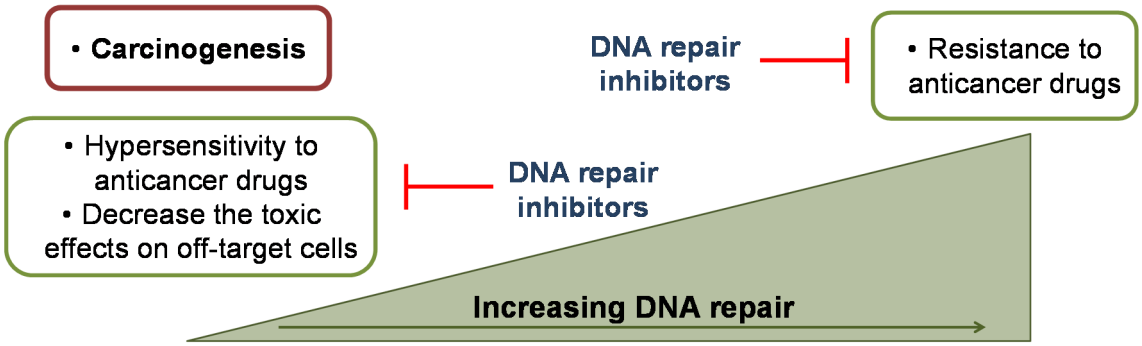


Figure 1.2 Rationale for the use of DNA repair inhibitors in cancer therapy. A jeopardised DNA repair increases the sensitivity to anticancer drugs and decreases the toxic side effects. An increased DNA repair activity is related with the resistance to anticancer agents. Targeting DNA repair is an opportunity to improve the cancer therapy outcomes [2,8,32].

1.2. THE BASE EXCISION REPAIR (BER) PATHWAY

The basic steps of the base excision repair pathway were first described in the 70s decade by Tomas Lindahl[1] after the identification of the *Escherichia coli* uracil-DNA glycosylase [9,36,37]. BER is known to play a critical role in the removal and replacement of several small non-bulky DNA lesions generated by oxidation, alkylation or deamination and the uracil from DNA. It is also the major DNA repair pathway responsible for repairing the apurinic/apyrimidinic (AP) sites spontaneously produced during the cellular metabolism or as intermediates of this pathway during the repair of

[1] Awarded with the Nobel Prize in Chemistry 2015 along with Paul Modrich and Aziz Sancar for mechanistic studies in the DNA repair field.

base modifications inflicted by endogenous or exogenous genotoxicants (Fig. 1.3). The SSBs can occur directly by the attack of ROS to the deoxyribose or indirectly upon repair being also a substrate of BER [9,10,38]. In this context, it is essential to highlight the pivotal role of the human APE1, a topic that will be thoroughly described in this and in the next sections of the present thesis.

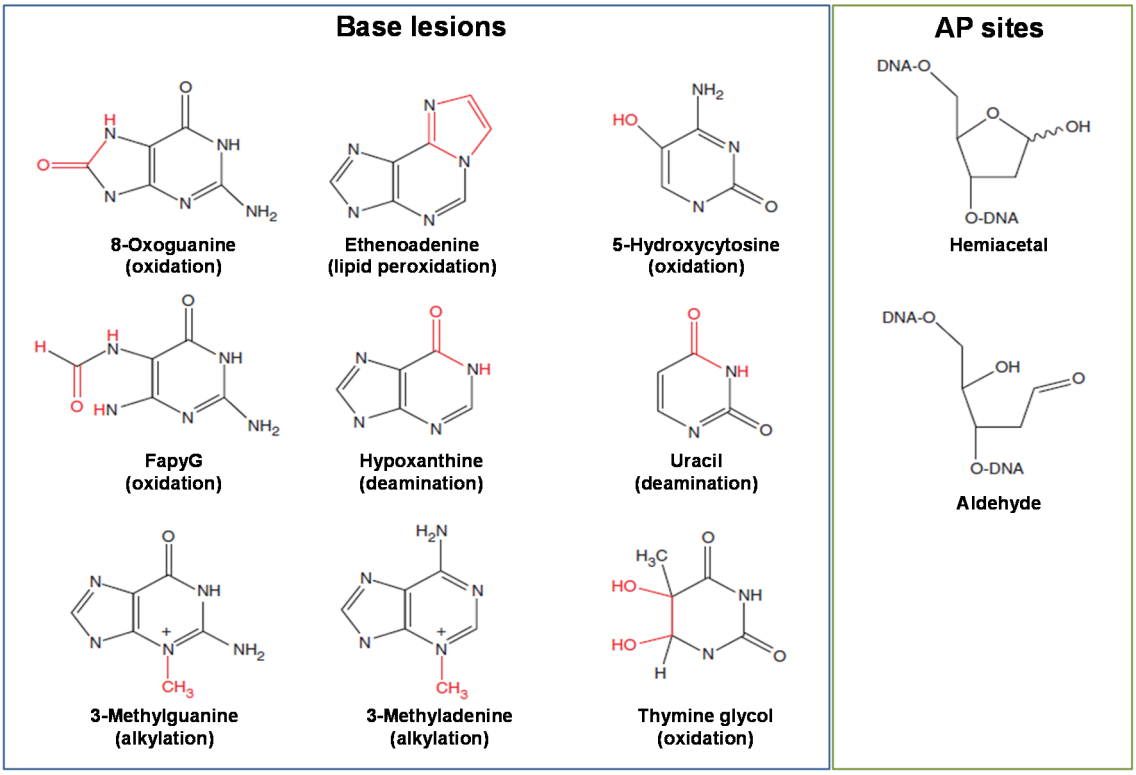


Figure 1.3 Chemical structures of some base lesions and apurinic/apyrimidinic (AP) sites commonly recognized and repaired by base excision repair pathway. Adapted from [9]. *Abbreviation: FapyG - 2,6-diamino-4-hydroxy-5-formamidopyrimidine.*

In general, BER comprises five coordinated enzymatic steps (Fig. 1.4) which include the recognition and excision of the damaged base by a DNA glycosylase, the incision of the AP site by APE1 or the AP lyase function of a bifunctional DNA glycosylase, the removal of the 5'- or 3'- terminal blocking groups, the DNA synthesis by a DNA polymerase for filling the resulting gap and, finally, the nick sealing carried out by a DNA ligase [9,10,39,40].

Firstly, a lesion-specific DNA glycosylase recognizes and removes the damaged or inappropriate base. The hydrolytic cleavage of the N-glycosidic bond linking the incorrect base and the DNA sugar-phosphate backbone carried out by DNA glycosylases produces an AP site [41,42]. The intact AP sites generated by the monofunctional DNA glycosylases (UNG, TDG, SMUG1, MUTYH, MBD4 and AAG/MPG) are further processed by APE1 which catalyses the hydrolysis of the

phosphodiester backbone 5' to the AP site producing a SSB with a deoxyribose phosphate (dRP) group at the 5' end and a 3'-OH termini. In the case of bifunctional DNA glycosylases (such as OGG1, NTHL1, NEIL1, NEIL2 and NEIL3) which also have an intrinsic AP lyase activity, the AP site incision is performed by the DNA glycosylase itself 3' to the AP site. The resulting SSB presents a 3'-non-ligatable group which should be removed to provide the proper substrate to be channeled to the following step of BER [10,39–41]. NTH1 and OGG1, primarily responsible for the excision of the oxidative lesions 8-oxo-7,8-dihydroguanine (8-oxoG) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG), have a weak lyase activity. They catalyse the AP site processing via a β-elimination reaction which creates an intermediate with a 5'-phosphate (5'-P) and a 3'-phospho-α,β-unsaturated aldehyde (3'-PUA) moiety. The 3'-blocking group is removed by APE1 intrinsic 3'-phosphodiesterase activity [10,39,40]. Regarding the NEIL glycosylase family responsible for the removal of oxidised pyrimidines, formamidopyrimidines, 5'-hydroxyuracil and urea from the DNA, the AP site incision is accomplished through a β, δ-elimination reaction with the formation of a SSB carrying a 3'-phospate (3'-P) group. Since the 3'-P moiety is not a suitable substrate for APE1, it can be removed by the phosphatase activity of polynucleotide kinase/phosphatase (PNKP) in an APE1-independent BER pathway [10,39,40,43,44]. However, the APE1-independent repair of AP sites is inefficient in the APE1 replacement during repair of the most commonly generated AP lesions.

The next steps of BER can proceed through two sub-pathways: the short-patch BER (SP-BER) or the long-patch BER (LP-BER) [10,39,40]. The aforementioned base excision and the cleaning of the terminal blocking groups are common to both sub-pathways. However, they diverge in the length of the DNA fragment to be replaced and in the subsets of enzymes involved in the DNA synthesis and nick sealing. The SP-BER seems to be the predominant BER sub-pathway and only one nucleotide is displaced to continue the repair. Then the DNA polymerase β (Polβ) lyase activity removes the 5'-dRP moiety and promotes the DNA synthesis from the 3'-OH group through its DNA polymerase activity to fill the existing gap. To complete the repair, the nick sealing is mediated by the integrated action of DNA ligase IIIα and the scaffold protein X-ray cross-complementing factor 1 (XRCC1) [10,39,40]. When 5' end cleaning lyase activity of Polβ is incapable to remove the 5'-blocking groups present in oxidised AP sites BER occurs through LP-BER [10,39,40]. For example, the C1'-oxidised 2'-deoxyribonolactone is repaired by LP-BER to avoid the formation of a DNA-protein cross-link with the Polβ [39,45]. LP-BER requires the strand displacement synthesis of a fragment usually with 2-12 nucleotides [10]. The DNA synthesis is mostly assigned to

the replicative DNA polymerases δ and ε (Polδ/ε) although Polβ may also participate in the initiation of LP-BER [46,47]. The replication factor C (RFC) and the proliferating cell nuclear antigen (PCNA) support the Polδ/ε and the flap endonuclease 1 (FEN1).

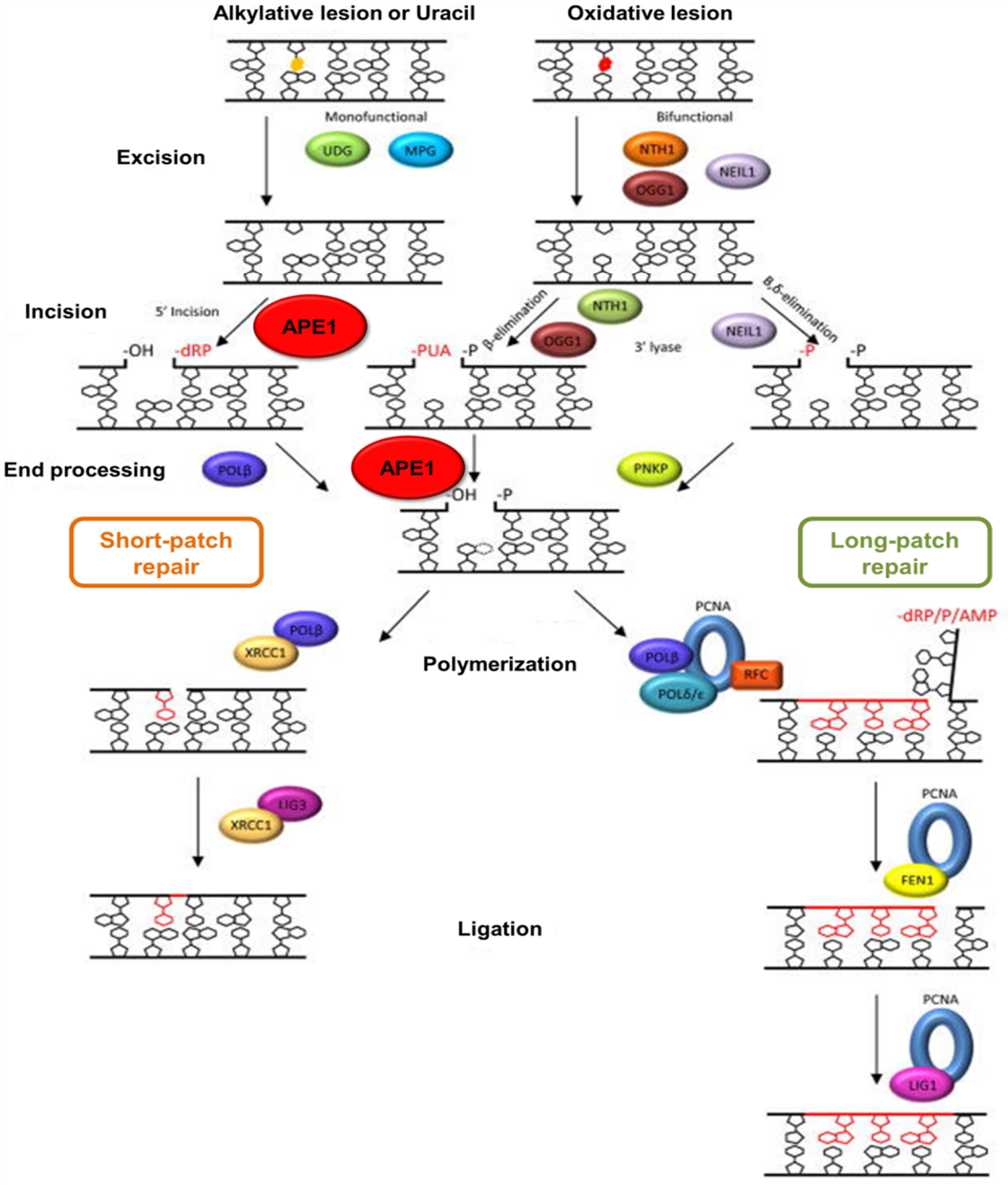


Figure 1.4 Overview of base excision repair (BER) sub-pathways: short-patch BER and long-patch BER. Both sub-pathways require the recognition and excision of the damaged base by a DNA glycosylase. After the strand incision at the abasic sites, 5'- and 3'- ends are processed by different enzymes according to the blocking groups. The repair may proceed through SP-BER or LP-BER to accomplish the DNA synthesis and ligation. Adapted from [40].

During the synthesis of the displaced oligonucleotide containing the 5'-sugar phosphate, PCNA functions as an accessory sliding clamp for Polδ/ε that is loaded into the DNA by RFC [10,39,48]. A flap intermediate is generated and further excised by the

structure-specific FEN1 [10,39,49]. Consequently, the substrate for DNA ligase I is created and double-stranded DNA is restored by nick sealing [10,39,40].

The selection of SP-BER or LP-BER remains to be fully clarified. The nature of the DNA damage, the intracellular levels of ATP, the protein-protein interactions, the cell cycle phase and the differentiation stage of cells have been reported to influence the sub-pathway choice.

The type of the DNA lesion determines the BER initiating DNA glycosylase and the 5'- and 3'-blocking moieties, which will define the DNA polymerase to be recruited and thus the BER sub-pathway [10,47,50–52]. Moreover, nick sealing reactions depend on the intracellular ATP availability to generate a covalent phosphodiester bond linking the 5'- and 3'-DNA strand ends. In the presence of high ATP levels the catalytic activity of the complex DNA ligase IIIα/XRCC1 is favored being the SP-BER the predominant mechanism. Under conditions of ATP depletion the DNA repair is shifted from SP-BER to LP-BER due to the promotion of strand displacement DNA synthesis by Polβ and allowing the DNA ligation by DNA ligase I [53,54].

In BER the intermediates are transferred to the next enzyme in a sequential and tightly orchestrated stepwise process to prevent the exposure of the cellular components to potentially harmful molecules [52]. Among the protein-protein interactions regulating the switch between SP-BER and LP-BER, XRCC1 and SSBs sensor proteins PARPs have a critical role in BER coordination [39,52]. XRCC1 is regarded as a scaffold protein interacting with several BER proteins to stimulate their recruitment for the SSBs, to promote their stabilisation and modulating their activities. In addition to DNA ligase IIIα [55], XRCC1 has been described to interact with proteins implied in different stages of both sub-pathways [56] such as the PCNA [57], APE1 [58], PNK [59,60], Polβ [61–63], NEILs [43,44,64], PARP1 and 2 [63,65,66]. The enrollment of PARP enzymes, especially PARP1, to the DNA-strand break is an early event of BER and probably occurs after the strand incision carried out by APE1 or a bifunctional DNA glycosylase. The activated proteins catalyse a nicotinamide adenine dinucleotide (NAD^+)-dependent synthesis of poly(ADP-ribose) chains which are a signal to the recruitment of multiple enzymes of BER for the damaged DNA site. PARPs functionally and physically interact with APE1, XRCC1, Polβ, PCNA and DNA ligase IIIα to boost the DNA repair. In the presence of an overwhelming DNA damage, PARPs can be overactivated leading to NAD^+ and ATP shortage and, consequently, triggering cell death by apoptosis or necrosis [67,68].

The cell cycle stage may also govern the SP-BER or LP-BER selection. The LP-BER shares proteins with the replicative machinery (e.g. FEN1, Polβ/δ/ε, DNA ligase I) suggesting its involvement in the repair of replication-associated DNA lesions. This hypothesis is supported by the detection at replication foci of protein complexes containing the aforementioned proteins. Thus the LP-BER can be more dominant at S/G2 cell cycle phases while a faster BER pathway such as SP-BER will be required in G1 phase (reviewed in [52]).

1.3. OVERVIEW OF HUMAN APURINIC/APYRIMIDINIC ENDONUCLEASE 1 (APE1) FUNCTIONS

APE1 is undoubtedly a key DNA repair enzyme that plays a crucial role in genome integrity as the major endonuclease in BER. Among the several physiological functions of APE1, its endonuclease activity and consequently its role as an upstream key player in SP-BER and LP-BER are of utmost importance as it was mentioned in the previous section. However, the therapeutic opportunities of targeting APE1 should not be restricted to the modulation of the DNA repair activity. APE1 is considered an ubiquitous multifunctional protein essential for the regulation of cellular response to oxidative stress and vital for the maintenance of genome stability and cell integrity [69–71]. Importantly, APE1, also designated redox effector factor 1 (Ref-1), has another major function since it independently acts as a reduction/oxidation signalling protein modulating the activation and DNA binding ability of several transcription factors which promote the expression of genes implicated in cell survival and in tumour promotion and progression (Fig. 1.5) [71–73]. Moreover, the understanding of the regulation as well as the role of distinct APE1 activities in cellular homeostasis is required to efficiently develop inhibitors of the different APE1 functions.

APE1 is a member of the Xth family of class II AP endonucleases due to its structural homology with the exonuclease III (Xth) enzyme of *Escherichia coli* [73–76]. In mammalian cells APE1 contributes for approximately 95% of the endonuclease activity in eukaryotic organisms [73,74]. APE1 is a monomeric α/β globular protein with 318 amino acid in length and a molecular weight of approximately 35 kDa [71,77]. Despite lacking the initial N-terminal fragment of 35 residues, the first X-ray crystal structure of APE1 revealed two main domains displaying similar topologies [77]. Both N-terminal and C-terminal domains have a six-stranded β-sheet encircled by α-helices folding together to form the four layered α/β sandwich structural core and resembling the motifs observed in the *E. coli* homologue exonuclease III and DNase I-like proteins

[71,77]. The first 40 N-terminal amino acids of APE1 structure have a disordered folding [78,79]. Notably, the C-terminal region is highly conserved among organisms from different kingdoms while the N-terminal fraction is almost restricted to mammals [80–82]. Noteworthy, the protein structural organization enables both DNA repair and redox activity to be physically and functionally independent since they are encoded by non-overlapping domains of APE1 [71,72,83]. While the catalytic site responsible for its endonuclease activity is located within the C-terminal domain, the redox regulatory function of APE1 is assigned to N-terminal portion which also harbours a complex bipartite nuclear localization signal (NLS) (Fig. 1.6) [71,81,83–85].

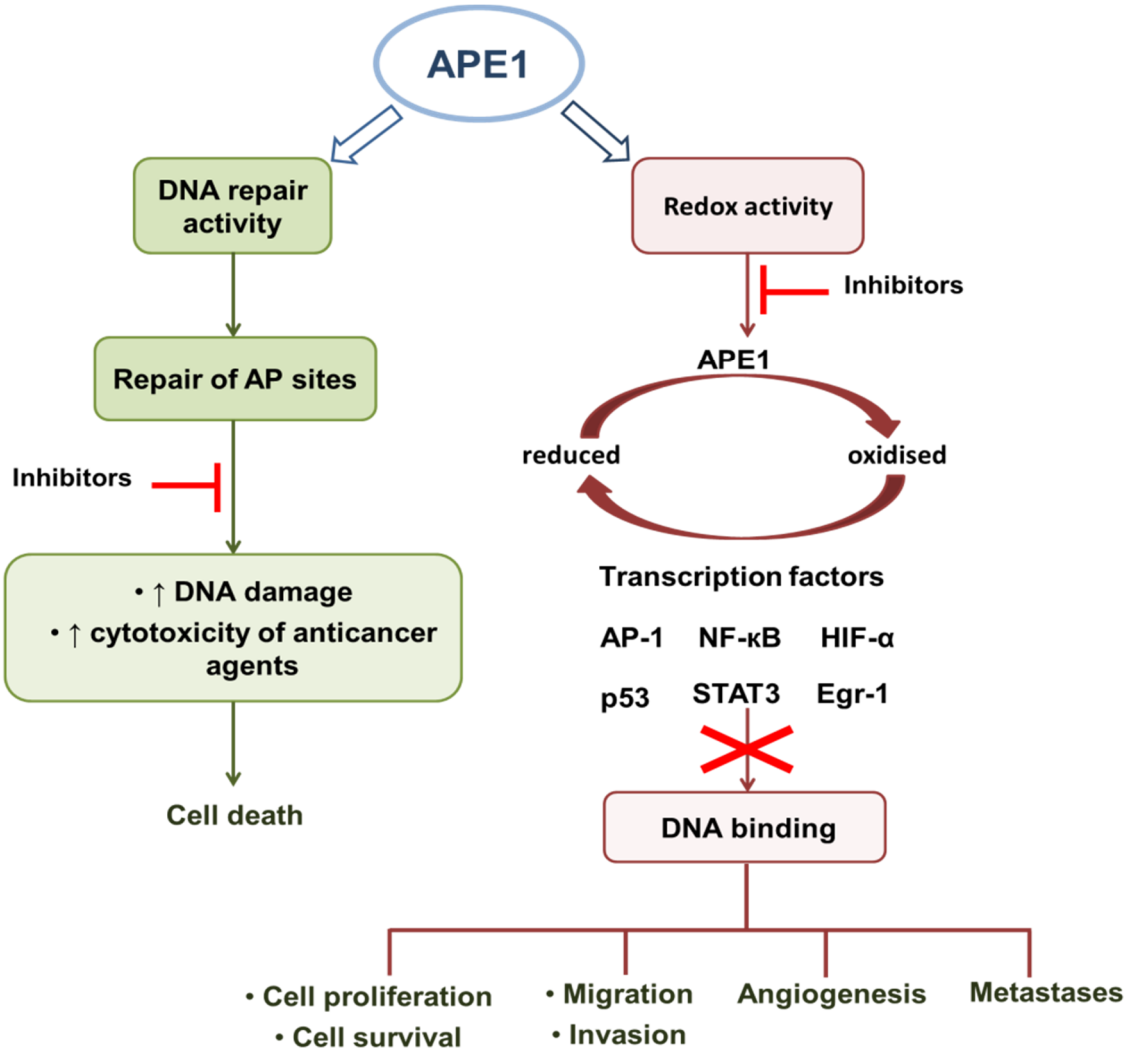


Figure 1.5 APE1 is a DNA repair protein with an independent redox function. APE1 participates in base excision repair pathway. The inhibition of APE1 endonuclease activity increases the DNA damage and might improve the cytotoxic effects of anticancer agents causing the tumour cells death. The inhibition of the reduction/oxidation signalling function of APE1 reduces the activation of several transcription factors modulating the expression of genes involved in cancer promotion and progression [71,72]. *Abbreviations: AP – apurinic/apyrimidinic; AP-1 – activator protein 1; Egr-1 – early growth response protein 1; HIF-α – hypoxia inducible factor 1 alpha; NF-κB – nuclear factor-kappa B; STAT3 – signal transducer and activator of transcription 3.*

The **DNA repair function** of APE1, particularly its endonuclease activity, is essential for the repair of toxic AP sites spontaneously generated, chemically induced or resulting as intermediate products in the enzymatic hydrolysis of DNA bases damaged by alkylating and oxidising agents (e.g. ROS) carried out during BER [86]. In the intracellular milieu, AP sites are an equilibrium mixture of four species corresponding 99% to a mixture of equal parts of the two hemiacetal enantiomers α- and β-2-deoxy-D-ribofuranose and approximately 1% to the ring-opened aldehyde and hydrated aldehyde forms [87]. The ring-opened forms are highly reactive providing a site for DNA cleavage by a β-elimination reaction [87,88]. If left unrepaired, non-coding AP sites can be cytotoxic and mutagenic threatening the integrity of cell function and survival [87,89]. The cytotoxicity of AP sites may result from the ability to induce replication forks stalling with the generation of SSBs which can be converted into DSBs after replication [87,89,90]. The ring-opened AP sites can also react with nuclear proteins yielding DNA-protein complexes which can hamper the DNA replication [87,89]. Another cytotoxic mechanism of AP sites may be related with the interference with topoisomerases DNA cleavage activity or the irreversible trapping of topoisomerase-DNA covalent complexes [87,89,91].

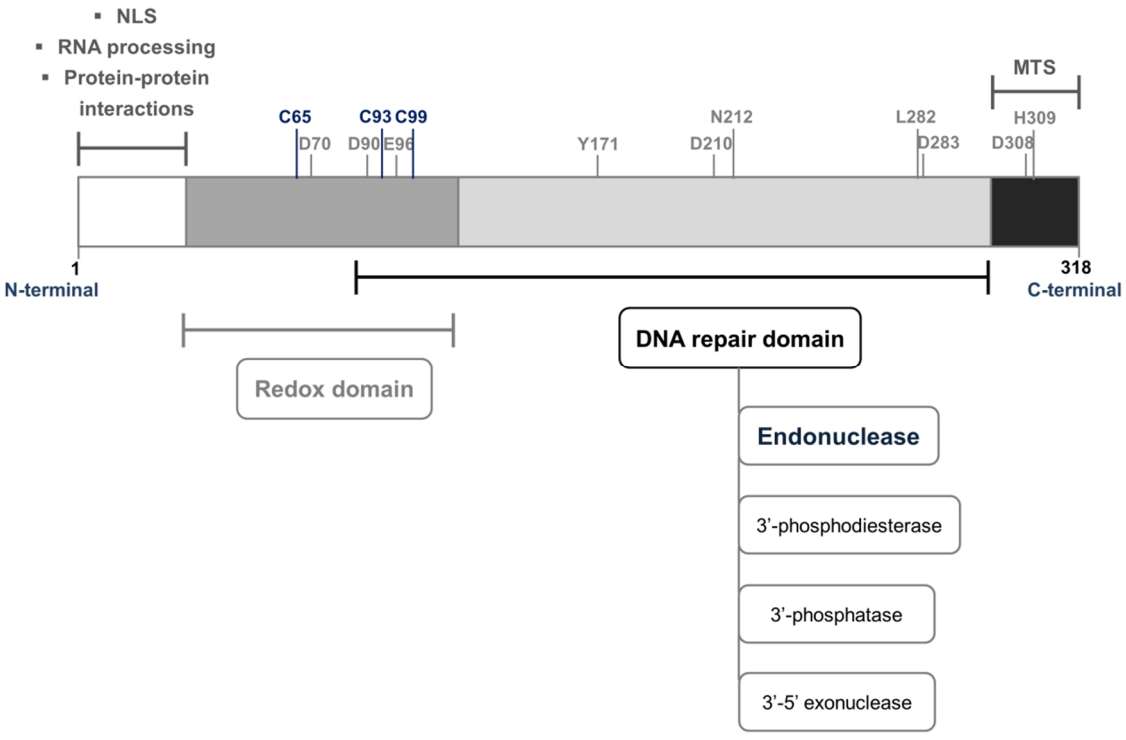


Figure 1.6 Schematic representation of the primary structure of human APE1. The active domains and their major functions are displayed. The essential residues for APE1 redox function are highlighted in blue and the amino acids involved in the endonuclease activity are depicted in grey. Adapted from [81,85,89]. *Abbreviations: NLS – nuclear localization signal; MTS – mitochondrial targeting sequence.*

The APE1 **endonuclease activity** is the major responsible for the recognition and repair of regular AP sites. After the recognition of the abasic nucleotide, APE1 performs the hydrolytic cleavage of the phosphodiester backbone 5' to the AP site in a Mg^{2+}-dependent reaction. A SSB containing a 3'-hydroxyl group and a dRP termini is generated. This DNA intermediate is channeled into one of the BER sub-pathways to complete the repair [71,73,89]. The APE1's active site that catalyses the endonucleolytic cleavage is located in the C-terminal domain in an hydrophobic pocket located at the top of the α/β sandwich and surrounded by loop regions [71,77,79].

Besides its primary role as an apurinic/apyrimidinic endonuclease, APE1 has also a **3'-phosphodiesterase** and a weak **3'-phosphatase activity** to remove non-conventional 3'-blocking groups generated by the AP lyase function of bifunctional DNA glycosylases or induced by IR, ROS or radiomimetic anticancer drugs, such as bleomycin. The 3'-phosphodiesterase activity eliminates the 3'-phosphoglycolate (3'-PG) group created by the fragmentation of the sugar moiety while the 3'-phosphatase activity removes 3'-phosphate residues to render a SSB with a 3'-OH termini which is required to continue BER [10,71,92]. APE1 also exhibits a **3'-5' exonuclease activity** although it had been suggested to be weaker than its endonuclease activity. The 3'-5' exonuclease function displays an important role in the removal of 3' mispaired nucleotides at nicked DNA providing a proofreading activity and ensuring the fidelity of the DNA repair during BER [89,93,94]. The APE1 exonuclease activity was also shown to remove the nucleoside analogues incorporated at the 3' end of the DNA strand and thus contributing for the therapeutic effects and toxicity of anticancer drugs such as troxacitabine, an unnatural L-deoxycytidine analogue [89,95]. The ability to remove the RNA strand from a RNA-DNA complex through a **RNase H activity** was also attributed to APE1 [71,89]. These repair functions seem to share the same active site reported for the APE1 endonuclease activity in the C-terminal domain [89,96].

In addition to the crucial roles in both BER sub-pathways, APE1 was also proposed to participate in the **nucleotide incision repair (NIR)** which requires the APE1 incision of the phosphodiester backbone immediately 5' to the oxidatively damaged base. Although NIR proceeds in a glycosylase-independent manner, BER and NIR have overlapping substrate specificities. Therefore, NIR can be an alternative pathway to BER for the repair of potential genotoxic oxidative DNA lesions [97]. Differently from the other repair functions, NIR activity has been suggested to be regulated by the N-terminal region of APE1 [97].

Regarding its DNA repair functions APE1 is preferentially located in the nucleus, although the extra-nuclear activities attributed to this protein require the intracellular trafficking to the cytoplasm. Mitochondria are one of the predominant APE1 cytoplasmic locations highlighting the importance of APE1 participation in BER of mitochondrial DNA which is subjected to high levels of oxidative stress during aerobic metabolism [89,98,99]. Although the mitochondrial translocation mechanism of APE1 is not clarified it requires the mitochondrial targeting sequence (MTS) identified in the C-terminal domain and corresponding to the sequence of residues 289-318 [100].

The N-terminal domain holds the **redox activity** of APE1. The mechanism probably involves a thiol-mediated redox reaction with a Cys residue from APE1 acting as nucleophilic residue to reduce the Cys residues located in the DNA binding domains or in other regulatory regions of the transcription factor. Therefore, APE1 operates as a redox signalling protein by modulating the activation and DNA binding of several transcription factors to gene target sequences involved in cell survival and stress response. Indirectly, the redox function of APE1 is an important regulator of gene expression [71,72,89].

Along with the aforementioned roles in the repair of ROS-induced DNA damage and redox signalling, APE1 suppressed intracellular oxidative stress and apoptosis in endothelial cells by modulating ROS production through the regulation of the Rac1-dependent activation of NADPH oxidase [101]. ROS, Rac1 and NADPH oxidase participate in several processes required for cell motility and migration probably through the regulation of the constant re-organization of actin cytoskeleton. Therefore, APE1 may be indirectly involved in cell migration by controlling the intracellular ROS levels which contribute to the modulation of the restructuring of actin filaments [71,101]. It was also demonstrated the ability of APE1 to control ROS generation via a negative regulation of Rac1 and NADPH oxidase during the *Helicobacter pylori* infection of gastric and intestinal epithelial cells [102].

APE1 has also an intrinsic **RNase H activity** although its ability to degrade the RNA from a DNA-RNA complex is relatively inefficient and the biological relevance is not well understood [103]. More recently, an **endoribonuclease activity** was also attributed to APE1 suggesting a potential function in the RNA quality control process and in the regulation of mRNA expression [104–106]. APE1 is capable of cleave damaged RNA including abasic single-stranded RNA [105,107,108]. The endonucleolytic activity of APE1 was also found to perform the cleavage of the coding region determinant of mRNA of *c-myc* proto-oncogene regulating its levels and half-life

in cells [105]. Although endoribonuclease and other nuclease activities of APE1 appear to share the same active site in the C-terminal domain, the mechanisms for incising injured RNA or DNA are not completely identical [107]. The N-terminal domain is also required for the stable binding of APE1 to RNA and to the interaction with proteins involved in ribosome biogenesis and ribosomal RNA (rRNA) cleansing processing [106]. The nucleolar protein nucleophosmin 1 (NPM1) is one of APE1 interacting partners. The formation of the complex NPM1-APE1 seems to have a stimulatory effect on the endonuclease activity of damaged DNA and an inhibitory effect on the endoribonuclease activity of APE1 probably by making unavailable the vital residues for APE1 binding to the RNA [106,109].

The physical and functional interactions of APE1 are not restricted to proteins involved in BER, such as DNA glycosylases, XRCC1, PCNA, FEN1, Polβ and PARP1, or in RNA metabolism [58,106,109–113]. In fact, the APE1 interactome network is wide, as reviewed in [71,114,115] and it encloses interactions with non-canonical proteins involved in APE1 redox chaperone activity (e.g. thioredoxin), cell death pathways (e.g. granzyme A and Bcl2), cytoskeleton rearrangement and cellular stress response signalling pathways (e.g. heat shock protein 70 (Hsp70)), among others [81,115–117]. The disordered N-terminal domain appears to mediate the APE1-protein interactions [71,82,106,114,115].

APE1 was also found to function as a ***trans*-acting factor** with a Ca^{2+}-dependent transcriptional repressor activity on the parathyroid hormone (PTH) [118]. High levels of PTH trigger an increase in extracellular Ca^{2+} which regulates the binding of a protein transcriptional complex containing APE1 to the negative calcium responsive elements (nCaRE-A and nCaRE-B) to inhibit the transcription of PTH gene [71,89,118]. Similarly, nCaRE-A and nCaRE-B elements were also identified in the human APE1 promoter gene suggesting that APE1 itself may integrate the transcriptional complex and regulate its expression through a negative feedback mechanism [71,89,119]. APE1 acetylation at N-terminal residues Lys6 and Lys7 seems to enhance its binding affinity to nCaRE elements of PTH promoter. APE1 endonuclease activity probably is not affected by this post-translational modification of mammalian APE1, since the best candidates to be acetyl acceptors are located in the N-terminal region of the protein [120,121]. Ca^{2+}-activated histone acetyltransferase p300 was proposed as the major responsible for APE1 acetylation, which is required for its co-repressor activity [120]. The acetylation of APE1 also appears to be required for the early growth response protein 1 (Egr-1)-mediated activation of phosphatase and tensin homolog (PTEN) expression [122]. More recently, acetylated APE1 was shown to stably interact with Y-

box-binding protein 1 (YB-1) enhancing the assembly, recruitment and binding of the complex containing APE1, YB-1 and p300, and promoting the loading of the basic transcription factor RNA polymerase II on the promoter of the multidrug resistance gene *MDR1* [123,124]. The acetylated APE1-mediated transcriptional activation of MDR1 has shown a potential role in the sensitivity of tumour cells to doxorubicin, cisplatin and etoposide [123,124].

Finally, other post-translational modifications have been reported for APE1, including phosphorylation, S-nitrosation and ubiquitination [71]. The *in vivo* biological significance of APE1 phosphorylation is not elucidated and *in vitro* studies were not able to understand its importance in gene regulation. While Yacoub *et al.* [125] revealed the ability of casein kinase II-mediated phosphorylation of APE1 to inactivate its endonuclease activity, Fritz *et al*. [126] found an enhancement in the redox activation of transcription factor activator protein 1 (AP-1) without affecting the endonucleolytic function. The S-nitrosation of the residues Cys93 and Cys310 may coordinate the APE1 translocation from the nucleus to the cytoplasm and the reversible S-glutathionylation of Cys99 seems to reduce the APE1 incision activity [127,128]. The N-terminal lysine residues are also the preferred ubiquitination sites which are regulated by the p53-mouse double minute 2 (MDM2) signalling pathway. APE1 ubiquitination may retain the BER protein in the cytoplasm and interfere with the interactions of APE1 with nucleic acids and other proteins [82,114,129]. Due to the pleiotropic nature of APE1, post-translational modifications may be crucial for the regulation of many biological activities being also potential targets for the APE1 functions modulation [71].

1.4. DNA REPAIR ACTIVITY OF APE1 AS A THERAPEUTIC TARGET IN CANCER

Several preclinical and clinical lines of evidence supported APE1 as a prospective target for cancer therapy and highlighted a potential role as predictive biomarker [16,71,73,130]. The targeting of APE1 DNA repair activity with small-molecule inhibitors appears to be a clinically useful strategy to counteract the resistance of tumour cells to conventional anticancer agents, namely IR, alkylating and antimetabolite drugs [16,72,73,89,131]. Moreover, the pharmacological inhibition of APE1 endonuclease activity may also be a promising personalised monotherapy approach since it can lead to synthetic lethality in tumours harbouring mutations in proteins associated with defects in HR [132,133]. In the following sections the main features of APE1 DNA

repair active site to consider in the development of APE1 inhibitors and the currently reported compounds with an inhibitory activity of DNA repair function will be addressed.

1.4.1. Structural insights into the DNA repair active site of APE1 in the context of drug discovery

The development of potent and effective small-molecule inhibitors of the APE1 endonuclease function requires the knowledge of the protein-ligand interactions and protein structure, namely the binding site and the stoichiometry of metal ion(s) present in the active site. Although the role of APE1 endonuclease activity in BER is recognized, the catalytic mechanism of the cleavage reaction is not completely elucidated.

The availability of numerous X-ray crystallographic structures of APE1 bound or unbound to DNA substrates enabled the identification of the active site with one or two metal ions depending on the acidic or neutral conditions used to solve the structures. The APE1 crystal structures obtained under an acidic pH commonly revealed a single metal ion in the active site (Protein Data Bank (PDB) codes: 1BIX, 1HD7, 4LND, 4IEM, 4QHE, 4QH9 and 5DFF) while crystallization at a neutral pH (PDB code: 1E9N) suggested that APE1 might bind to two divalent metal ions in its active site, raising the controversy [77,134–138].

The first crystal structure of APE1 was solved by Gorman *et al.* [77] at a resolution of 2.2 Å and deposited in PDB with the code 1BIX. Although lacking the 35 N-terminal amino acids, this truncated APE1 was previously shown to be fully active. In this structure, the active site was found within a hydrophobic pocket where the hydrogen bonds established between several amino acids, namely the residues His309, Asp283, Thr265, Tyr171, Glu96, Asn68, Asp210, Asp70 and Asn212, form a hydrogen bond network [77]. A single samarium ion bound to the side chain of Glu96 and Asp70 was also present in close proximity to the metal ion being a potential participant in the metal binding [77]. APE1 endonuclease activity was shown to require the presence of a divalent metal ion, presumably, the magnesium ion (Mg^{2+}) [139]. In this case and as a consequence of the experimental conditions used to obtain APE1 crystals with good quality, samarium ion is probably occupying the location of the more physiological Mg^{2+} ion in the APE1 active site [77]. These structural findings supported a catalytic mechanism with His309 abstracting a proton from a water molecule to generate the nucleophile able to attack the DNA phosphodiester bond while Asp283 interacts with His309 to orientate and stabilise the positive charged intermediate. The binding of the

metal ion to Glu96 could also be required to the stabilization of the transition state intermediate [77].

The determination of co-crystal structures of APE1 bound to DNA substrates containing synthetic AP sites (PDB codes: 1DEW, 1DE8 and 1DE9) and representing pre- and post-cleavage complexes displayed the molecular steps for substrate recognition [79]. The APE1 active site is rigid and pre-formed for the recognition of the DNA substrate, which undergoes conformational changes to allow the insertion of APE1 residues into the major and minor grooves of DNA helix. The binding of APE1 to DNA requires a bending of the DNA of approximately 35° which might assist the displacement of the DNA glycosylase and channeling to APE1 [79]. Arg177 and Met270 insertion in the major and minor groove, respectively, restraints the AP-DNA into APE1 active site [79]. Mol *et al.* [79] proposed that the APE1 hydrophobic pocket is lined by Phe266, Trp280 and Leu282 allowing the specific binding of the α-anomers of extrahelical AP sites generated by DNA glycosylases and avoiding the release of toxic intermediates during the initial steps of BER. According to [79], the divalent metal ion and the interactions with APE1 active site residues Trp280, Asn222, Asn226, Asn229, Asn212, Asn174 and His309 orientate the flipped-out AP-DNA. The phosphodiester bond cleavage involves an hydroxyl nucleophile activated by the buried Asp210 with the generation of a pentavalent transition state stabilised by the metal ion coordinated by Asp70 and Glu96, which also stabilises the leaving group of the cleavage reaction. The collapse of the transition state leads to the cleavage of the phosphodiester bond 5' to the AP site [79].

Beernink *et al.* [134] described the structures of two new crystal forms of unbound APE1. The structure solved at an acid pH (PDB code: 1HD7) revealed only one metal binding site designated as site A, while APE1 crystals grown at a neutral pH (PDB code: 1E9N), corresponding to a physiological pH for APE1 endonuclease activity, showed two metal binding sites [134,140]. The second divalent metal binding site was named site B [134]. The site A is present in all crystallographic structures previously identified and the metal ion is coordinated by carboxylate groups of residues Asp70 and Glu96 and with a water molecule which interacts with Asn68 and Asp308 through hydrogen bonds [77,79,134]. On the other hand, site B is formed by the side-chains of Asp210, Asp212 and His309. The divalent metal ion in site B also interacts with a water molecule bound to Tyr171 [134]. The carboxylate groups of Glu96 and Asp210 interact with the metal ions. According to the structures showing two metal ions in the catalytic active site, Beernink *et al.* [134] proposed a two metal-assisted catalytic mechanism for APE1. In this mechanism, a water molecule is probably activated by the Mg^{2+} ion

located at site B and generates an hydroxyl ion to perform the nucleophilic attack to the 5'-P termini of the AP site [134]. The Mg^{2+} bound to site A coordinates and stabilises the 3' leaving group [134]. The presence of two metal binding sites was further supported by biochemical assays yielding biphasic inhibition curves for APE1 [134]. Based on experimental evidences and molecular dynamic simulations Oezguen *et al.* [141,142] suggested an alternative "moving metal mechanism". The authors proposed that APE1 has only one metal ion which could move between the two sites during the cleavage reaction [141]. In the pre-cleavage complex the divalent metal ion interacts with the residues of the catalytically more active and buried site B [141]. After the reaction, the metal ion moves to site A to facilitate the retention of the cleaved product and the binding of the next BER enzyme which should process the DNA [141].

Nuclear magnetic resonance (NMR) spectroscopy was also used as an alternative method to X-ray crystallography to describe the APE1 metal binding site(s) [143]. A single specific Mg^{2+} binding site was identified in a functional APE1-DNA complex consistent with site A [134,143]. The crystal structure of APE1 bound to a double-stranded DNA containing an AP site mimetic (PDB code: 4IEM) also showed only one Mg^{2+} ion in the active site directly coordinated by Glu96, 3'-ribose oxygen, phosphate from DNA and three water molecules in a tetrahedral arrangement [135]. These findings were recapitulated by a high resolution (1.92 Å) crystal structure of DNA-free APE1 with a single Mg^{2+} bound in the active site (PDB code: 4LND) obtained by Manvilla *et al.* [136]. The position of Mg^{2+} is similar to the previously described site A [134,136] and the ion is coordinated in an octahedral geometry by carboxylate groups of Asp70 and Glu96 and four water molecules [136]. Both methods and structures had no evidences of a metal ion in site B [135,136,143]. He *et al.* [137] corroborated the presence of a pre-formed single metal binding site in APE1 and suggested the initial capture of the metal ion in the active site. In addition to Asp70 and Glu96, Asp308 may also participate in the stabilisation of the Mg^{2+} ion. Similarly to the findings of Lipton *et al.* [143], the crystal structure with the PDB code 4QHE also showed a disordered Mg^{2+} ion, which exhibited two partially occupied binding sites due to the ability of Glu96 to present different conformations [137]. These evidences highlight the structural plasticity of the active site during catalysis [137].

The most recently catalytic mechanism interpretation was proposed by Freudenthal *et al.* [138] by capturing key snapshots of APE1-DNA structures (Fig. 1.7). Overall, AP site is flipped by APE1 into the active site to position the DNA for the cleavage reaction. In the precleavage (or ground) state, a single metal ion is bound to Asp70, Glu96 and a water molecule, while another water molecule is positioned through the interactions

with Asn212 and Asp210 for the nucleophilic attack to the phosphorus atom 5' to the AP site. A pentacovalent transition state-intermediate is generated and stabilised by Mg^{2+}, Tyr171, His309, Asp210 and Asn212. The active site Mg^{2+} coordinates with an oxygen from the DNA product and with an oxygen of the 3' leaving group. In the final product state, Asn212 rotates acting as an hydrogen-bond donor to stabilise the product.

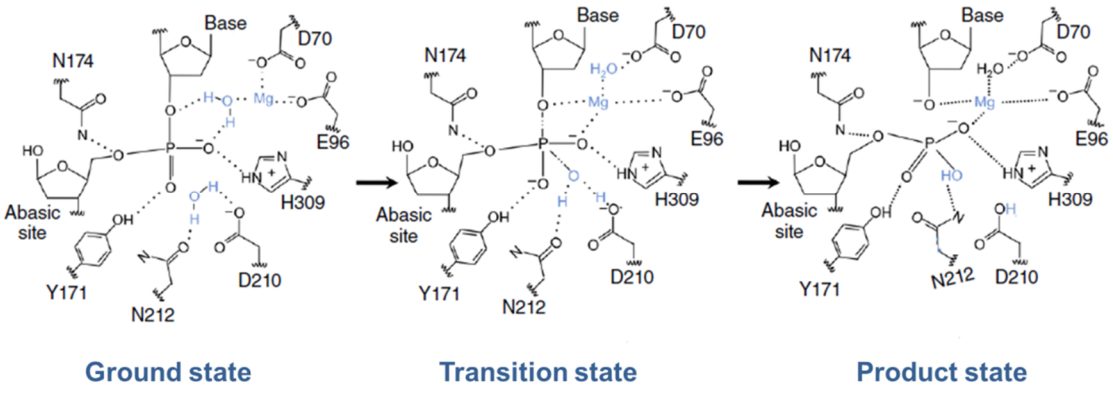


Figure 1.7 Scheme of the suggested catalytic mechanism of APE1 endonuclease activity. The mechanism interpretation was based on the structural snapshots of DNA-bound APE1 crystal structures obtained by Freudenthal *et al.* [138]. The transition state was predicted from the ground and product state structures. Adapted from [138].

1.4.2. Currently available small-molecule inhibitors of APE1 DNA repair activity

The modulation of the DNA repair function of APE1 has been widely studied in cancer therapy and several authors have been dedicated to the identification and development of small-molecule inhibitors of the DNA repair activity of APE1.

The most studied inhibitor of APE1 endonuclease function is methoxyamine hydrochloride (MX), also known as TRC102 (Fig. 1.8) [16]. MX was the first APE1 inhibitor to be identified and, as aforementioned, the first to be evaluated in human clinical trials in combination with alkylating or antimetabolite drugs. MX is an alkoxyamine derivative that indirectly inhibits the DNA repair function of APE1 [144,145]. The reaction of MX with the aldehyde group of the ring-open form of deoxyribose moiety present in the AP site after the removal of the damaged nucleotide by the DNA glycosylase creates a blocked AP site [131,144,145]. The covalently bound MX-AP sites are refractory to the APE1 endonuclease activity inhibiting BER progression and inducing the accumulation of cytotoxic AP sites in cells [131,145]. Moreover, the stable adducted MX-AP sites also target the topoisomerase II and promote the formation of DSBs by acting as topoisomerase II poisons which lead to cell

death [145]. *In vitro* and *in vivo* preclinical studies showed the MX sensitisation towards the cytotoxic effects of TMZ [145–149], 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) [150], pemetrexed [151] and 5-iodo-2'-deoxyuridine (IdUrd) [152] as well as the potentiation of IdUrd-mediated radiosensitisation [153] in several solid tumour models. These results provided the proof-of-concept to conduct clinical trials with MX as adjuvant therapy of anticancer agents. The already completed phase I clinical trial of oral administered MX (TRC102) in combination with pemetrexed in patients with advanced refractory cancer showed that the APE1 inhibitor was well-tolerated at clinically relevant doses [154]. A phase I clinical trial of the combination of MX (TRC102) with fludarabine phosphate in patients with relapsed or refractory haematologic malignancies was also completed showing no dose limiting toxicity in low-grade lymphoma and chronic lymphocytic leukemia with relapses after fludarabine-containing regimens [155]. Clinical trials with the combination of MX with TMZ or cisplatin and pemetrexed are also planned (Table 1.2)

Although the potential of MX as an inhibitor of APE1 endonuclease function, many efforts have been made to develop direct inhibitors of this enzyme. In fact, direct enzyme inhibitors are considered more specific with a subsequent decrease of secondary targets which probably reduces the therapy-related side effects [131].

7-nitro-1*H*-indole-2-carboxylic acid (CRT0044876) (Fig. 1.8) was identified in a high-throughput screening (HTS) assay of a commercially available library of "drug-like" compounds as a direct inhibitor of APE1 with an IC_{50} of approximately 3 µM [156]. CRT0044876 revealed promising *in vitro* results showing an increase in the cytotoxicity of methyl methanesulfonate (MMS) and 5-hydroxymethyl-2'-deoxyuridine [156]. However, some authors were not able to reproduce the effects reported by Madhusudan *et al.* [156] for this compound [73].

Lucanthone (Fig. 1.8) was shown to act as a nonspecific direct inhibitor of APE1 since topoisomerase II is also a target of lucanthone which is also able to intercalate into the DNA [157,158]. Nevertheless, the interaction of lucanthone with other cellular targets and the resultant toxicity prevented its therapeutic use as APE1 inhibitor [131,157,158].

Different strategies have been used to identify novel direct small-molecules of APE1 DNA repair activity including the HTS of commercially available chemical libraries [156,159–162], the drug design approaches based on structure-activity relationship studies [163,164], ligand-based pharmacophore models [164–166] and structure-based virtual screening (SBVS) as well as molecular docking studies [165,167–169]. Consequently, numerous molecules have been suggested to directly inhibit APE1

endonuclease activity (reviewed in [85]). Although structurally diverse (Fig. 1.8), the majority of the already described putative APE1 inhibitors share some basic chemical features namely a) an hydrophobic core, b) hydrogen-bond acceptors and c) negatively ionisable groups to anchor compounds to the active site residues of APE1.

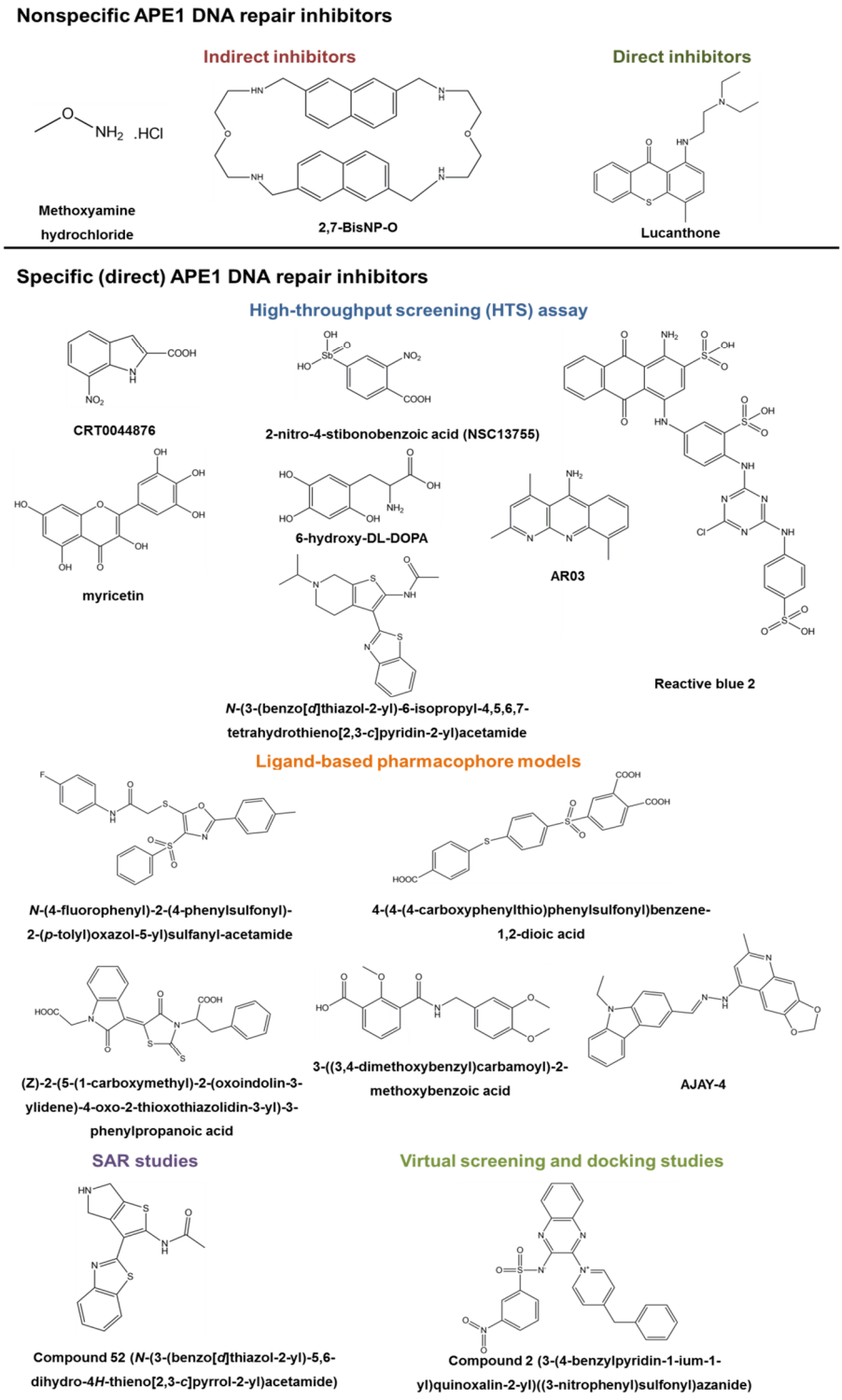


Figure 1.8 Structures of several reported inhibitors of APE1 DNA repair activity. The strategies adopted to identify each compound are also presented. These compounds were described in [156,158–163,165–170].

The approach adopted by Mohammed *et al*. [165] comprised the construction of pharmacophore models based on the compound CRT0044876 and prototypical molecular scaffolds designed to fit into the APE1 ligand-binding site. These structures were used to perform a SBVS study and docking analyses to select potential bioactive molecules [165]. The biochemical and cell-based assays in melanoma, glioblastoma and HUVEC endothelial cell lines in combination with MMS, TMZ and doxorubicin (Dox) led to the identification of *N*-(4-fluorophenyl)-2-(4-phenylsulfonyl)-2-(*p*-tolyl)oxazol-5-yl)sulfanyl-acetamide (Fig. 1.8) as a promising inhibitor of APE1 [165]. A similar strategy involving a receptor-based virtual screening and a docking study contributed to the identification of a compound containing a quinoxaline core which can be a scaffold for novel small-molecule inhibitors of APE1 [168].

Table 1.2 Clinical trials completed or planned with methoxyamine (MX) in cancer.

Tumour(s)	Combination therapy agent(s)	Phase of clinical trial[1]	Status of clinical trial	References
Advanced refractory solid tumours	Pemetrexed	Phase I	Completed	[154]
Relapsed or refractory haematological tumours	Fludarabine phosphate	Phase I	Completed	[155]
Lymphomas and relapsed solid tumours	Temozolomide	Phase I	Recruiting	www.traconpharma.com
Recurrent glioblastoma	Temozolomide	Phase II	Recruiting	www.traconpharma.com
Advanced solid tumours and refractory mesothelioma	Cisplatin and pemetrexed	Phase I/II	Recruiting	www.traconpharma.com
Stage IIIA-IV non-small cell lung cancer	Cisplatin, pemetrexed and radiation	Phase I	Recruiting	www.traconpharma.com
Advanced solid tumours	Temozolomide	Phase I	Recruiting	www.traconpharma.com

[1]As of 3rd July 2016, https://clinicaltrials.gov

The arylstibonic acids identified by Seiple *et al.* [162] are among the most potent inhibitors of APE1 in biochemical assays, being active in the low nanomolar range and inhibiting the enzyme activity by a partial mixed-type mechanism due to its ability to

bind to both the enzyme and the enzyme-substrate complex. However, these compounds did not show an increase of the toxicity of DNA-damaging agents in cell culture suggesting that issues of permeability might hinder their entry into the cells [162]. Moreover, carboxylate and stibonate groups appear to be positioned in the APE1 binding pocket similarly to the arrangement of the 3' and 5' phosphates of a DNA strand containing an AP site. This suggests that arylstibonic acids may mimic the phosphate groups of APE1 substrate [162]. This finding indicates that these compounds may be capable of binding other DNA-processing proteins disclosing a possible lack of specificity for APE1 [162].

Using a set of three-dimensional (3-D) pharmacophore models based on the interactions between APE1 and the abasic DNA, Zawahir *et al.* [166] reported a series of active compounds containing at least two terminal negatively ionisable chemical functions or bioisostere groups of negatively ionisable features. This highlights the requirement of these groups to the recognition of the potential inhibitors by APE1 and the subsequent inhibition of its DNA repair function [166].

The HTS assay performed by Simeonov *et al.* [160] revealed numerous and structurally diverse compounds as APE1 inhibitors. The aurintricarboxylic acid was the most potent hit. Although being active at low nanomolar concentrations it may form a stable radical homopolymer and inhibit other RNA- and DNA-processing enzymes [160]. Consequently, it was discarded from further analyses. Moreover, the most promising bioactive molecules were Reactive Blue 2, 6-hydroxy-DL-DOPA and myricetin (Fig. 1.8) which were already reported to have other molecular targets being non-specific inhibitors of APE1 endonuclease activity [160]. More recently, these authors performed the largest-scale HTS assay already described to identify novel inhibitors of APE1 and uncovered several potential scaffolds that can be chemically optimised [161]. In fact, one of these compounds was further used as lead chemotype in the first reported medicinal chemistry optimization campaign of a potential APE1 inhibitor [163]. The structure-activity relationship studies resulted in the synthesis of other possible APE1 inhibitors [163]. The molecule termed as compound 52 (Fig. 1.8) was more active than the lead compound (*N*-(3-(benzo[*d*]thiazol-2-yl)-6-isopropyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridin-2-yl)acetamide) in the biochemical assays [163]. Thus, compound 52 was also tested in cellular models and pharmacokinetic properties of both the lead and the optimized compound were evaluated in *in vitro* and *in vivo* models [163]. Although both compounds showed favourable solubility and permeability properties in *in vitro* models, compound 52 appeared to be rapidly metabolized by mouse liver microsomes. *In vivo*, compound 52 presented a more promising

cytotoxicity profile while the lead compound was more efficient to cross the blood-brain barrier [163].

A quinoline derivative designated as AR03 (Fig. 1.8) and identified in a HTS assay performed by Bapat *et al.* [159] appeared to be a specific APE1 inhibitor in the low micromolar range. It revealed also a sensitising effect of the cytotoxicity of MMS and TMZ in the SF767 glioblastoma cell line [159].

Several 3-carbamoylbenzoic acid derivatives (Fig. 1.8) were designed based on pharmacophore models previously reported and tested by Aiello *et al.* [164] showing APE1 catalytic activity inhibition in the micromolar range. Structure-activity relationship studies emphasised the role of 3-carbamoylbenzoic acid lead scaffold in the development of a new class of APE1 inhibitors [164].

Srinivasan *et al.* [167] also reported a putative APE1 inhibitor active in biochemical assays at nanomolar concentrations. This compound has a 2-methyl-4-amino-6,7-dioxolo-quinoline structure (Fig. 1.8) and potentiated the toxicity of a DNA-damaging agent that selectively induces N3-methyladenine lesions in T98G glioma cells. In addition, this compound was also tested in the NCI-60 cell line panel and revealed a synergic effect in SK-MEL-5 melanoma cell line exposed also to vemurafenib, a V600E-mutated B-type Raf (B-Raf) kinase targeting drug [169].

Although the advances towards the discovery of inhibitors of APE1 endonuclease activity, the abovementioned compounds still lack the potential to be translated to the clinical setting. In view of the increasing evidence supporting APE1 DNA repair function as an attractive therapeutic target, the development of pharmacologically active APE1 inhibitors to be used in cancer chemotherapy and to allow the understanding of the biological role of different functions of this enzyme remains a challenge [71,89].

1.5. REDOX FUNCTION OF APE1 AS A THERAPEUTIC TARGET IN CANCER

The maintenance of ROS homeostasis is required for the normal cell function. In non-tumour cells, a small increase in the basal low levels of ROS has a proliferative effect [171,172]. Regarding tumour cells, an increase in intracellular ROS levels can inhibit cell proliferation and induce cell death [171,172]. These differences are explained by tumour cells ability to adapt to higher levels of ROS [173,174]. Actually, tumour cells are under a persistent oxidative stress due to their high metabolic rate [171,173]. In general, tumour cells have low antioxidant defences and higher basal ROS level than non-tumour cells which are mitogenic and promote tumour growth [173]. Since basal

ROS concentrations in tumour cells are frequently close to the toxicity threshold a further increase in ROS levels can be pro-apoptotic [171].

Several conventional anticancer agents, including anthracyclines, taxanes, platinum-based drugs and IR, are known to generate ROS [172,175–179]. However, the opportunities of redox-based therapeutic approaches in cancer are not restricted to the use of anticancer agents in combination with pro-oxidants drugs to induce oxidative stress leading to DNA damage and cell death. A redox-based strategy also includes the modulation of antioxidant systems to counteract the toxicity of current available chemotherapeutic drugs [180]. For example, the life-threatening cardiotoxicity induced by the anthracycline Dox has been attributed to a ROS-dependent mechanism and antioxidant enzyme mimetics as well as intracellular metal chelators have been studied to improve the therapeutic index of the anticancer drug without affecting its chemotherapeutic effect [180–182].

Moreover, ROS are essential players in the cellular signal transduction pathways being required to the initiation of redox signalling cascades [183,184]. Upon the activation of a given pathway a series of coordinated redox reactions at the thiol groups of protein cysteine residues occur to convey the signal that will elicit the cell response [183,184]. ROS have been associated to the regulation of pathways involved in tumour cell survival, proliferation, invasion, migration and in angiogenesis [183–185]. Therefore, targeting of redox proteins of the signal transduction cascades is an attractive therapeutic alternative to disrupt redox signalling networks in tumour cells and abrogate important steps of cancer progression. In view of this, the redox activity of the multifunctional APE1 protein is an emerging upstream target for the modulation of several redox signalling pathways fundamental for cancer development [72,183–185].

1.5.1. APE1 in redox signalling

Mammalian APE1 can also function as a nuclear reduction/oxidation signalling factor and thus modulates the transcription of multiple genes involved in survival, proliferation and cancer promotion. Nuclear factor-kappa B (NF-κB), AP-1, hypoxia-inducible factor 1 alpha (HIF-1α), p53, Egr-1, signal transducer and activator of transcription 3 (STAT3), cAMP response element-binding protein (CREB) as well as tissue-specific proteins (e.g. paired box protein Pax-5 and -8) are some of the transcription factors under APE1 redox-mediated activation [71,72,186–191].

A redox mechanism has been proposed to involve the reduction carried out by APE1 of critical oxidised cysteine residues on the DNA-binding domains and/or located within

the regulatory regions of the transcription factors enhancing their affinity and binding to DNA [71,72]. In a typical thiol-disulfide exchange mechanism the attack of the nucleophilic cysteine to the disulfide bond of the inactive transcription factor results in the generation of a disulfide bond between the redox factor and the transcription factor. This bond has to be further resolved by an additional cysteine residue of the redox factor in order to generate the reduced transcription factor and the oxidised redox protein (Fig. 1.9) [71,72,81].

APE1 has seven cysteine residues in its structure and three are located in the N-terminal redox domain (Cys65, Cys93 and Cys99). Cys65 has been suggested as the nucleophile which reduces the transcription factors since its substitution by the amino acid alanine renders a redox inactive APE1 [192,193]. Moreover, the redox function and Cys65 are both present only in mammalian APE1 proteins supporting the critical role of Cys65 in APE1 redox activity [80].

Cys93 and/or Cys99 may be the residues involved in the resolution of the disulfide bond established between Cys65 and the transcription factor (Fig. 1.9). In fact, the substitution of Cys93 by alanine resulted in a decrease of the redox activity while the double substitution of Cys93 and Cys99 provided a redox inactive mutant APE1 protein as evaluated by an electrophoretic mobility shift assay (EMSA) [193].

Cys65 is a buried residue in APE1 structure being inaccessible to interact with the target transcription factor. Similarly, the position of Cys93 and the distance to Cys65 hampers the formation of a disulfide bond between these APE1 residues. In view of this, it was found that the redox mechanism requires a conformational alteration of APE1 to a partially or locally unfolded state allowing the exposure of the critical residues [80,193,194].

However, the mechanism of APE1 redox activity is not completely clarified. It has been suggested that the formation of additional disulfide bonds might occur and a third thiol-containing protein might also be implicated in the regeneration of oxidised APE1 [71,72,193]. In fact, thioredoxin was shown to interact with APE1 and the thioredoxin/thioredoxin reductase pathway also cooperates with APE1 in the control of p53 basal activity (Fig. 1.9) [81,195,196].

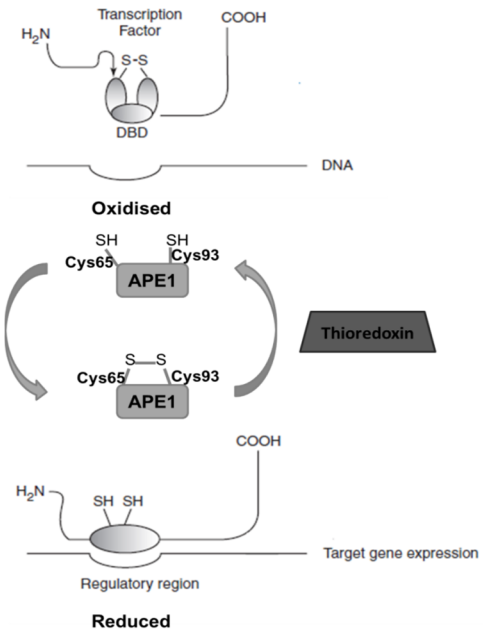


Figure 1.9 Scheme of the hypothesised mechanism of activation of transcription factors by APE1 redox function. Cysteines at position 65 and 93 are required for the reduction-oxidation reaction that reduces the transcription factors. Thioredoxin might be the third thiol-containing protein that regenerates the previously oxidised APE1 re-establishing its redox activity. Adapted from [81]. *Abbreviations: DBD – DNA-binding domain.*

1.5.2. Currently available small-molecule inhibitors of APE1 redox function

The APE1 redox activity participates in the downstream activation of transcription factors from several signal transduction pathways. Targeting of APE1 redox function may modulate different processes required for cancer promotion and progression. The currently available small-molecule inhibitors of APE1 redox function are presented in Fig. 1.10.

The compound (2E)-2-[(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)methylene] undecanoic acid, also known as E3330 (Fig. 1.10), was initially suggested to be an anti-inflammatory drug and an inhibitor of NF-κB DNA-binding activity [197–199]. The inability of E3330 to affect the degradation of IκBα, the nuclear translocation of NF-κB or the phosphorylation of the p65 subunit leads to the hypothesis that a redox nuclear cofactor should be required for NF-κB activity and act as the target of the quinone derivative E3330 [199]. APE1 has been shown to interact and activate NF-κB [186] and Shimizu *et al.* [200] identified APE1 as the protein being specifically inhibited by E3330.

The nature of the interaction of E3330 with APE1 has been widely studied in an attempt to understand the mechanism of action of the redox inhibitor. The binding of

E3330 to a partially unfolded conformation of APE1 was suggested by Su *et al*. [194]. In addition to protein stabilization, the establishment of the interaction APE1-E3330 allows the exposure of buried Cys residues essential for the redox activation of the transcription factors. E3330 might also induce the reversible activation of a Cys residue which will be more vulnerable to the nucleophilic attack by a reduced Cys of the protein. Therefore, E3330 increases the disulfide bond formation probably between Cys65 and Cys93 of APE1 decreasing the redox active molecules of the enzyme [193,194].

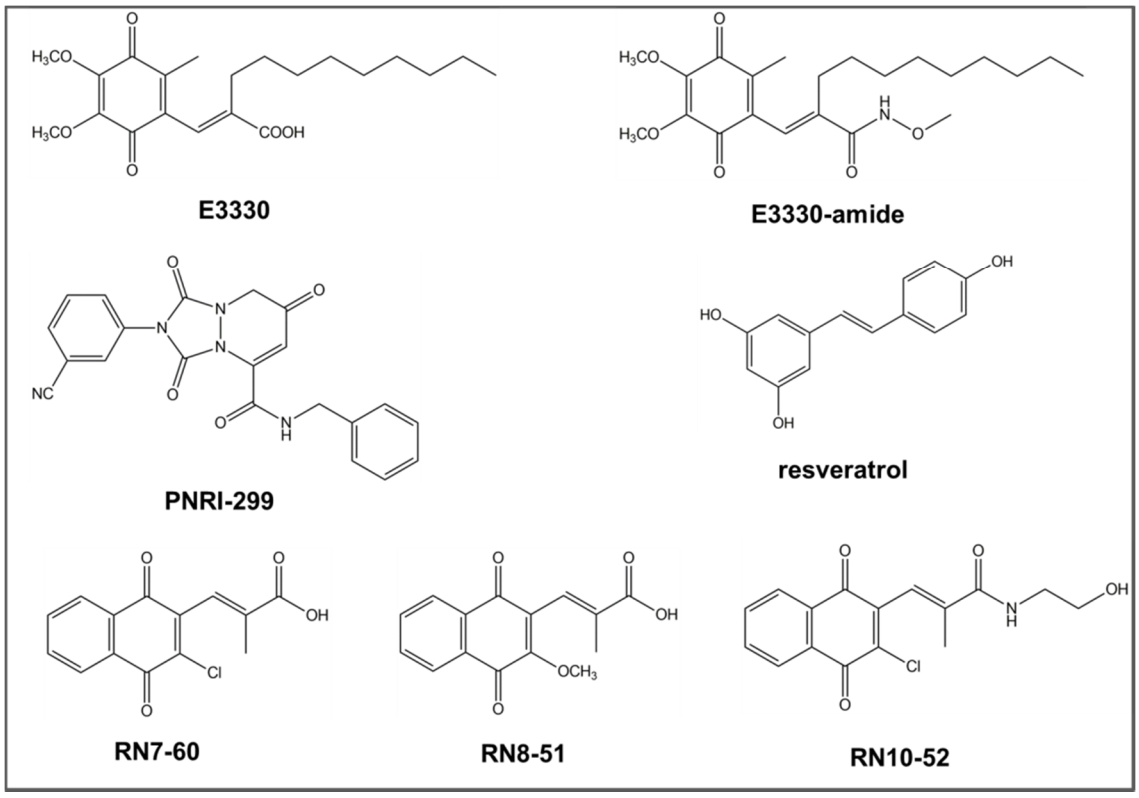


Figure 1.10 Structures of APE1 redox inhibitors. These compounds were described in [200–205].

The redox inhibitor binding site in APE1 has been postulated to be located near Cys65 which has been considered a vital residue for APE1 redox activity. Using a NMR shift assay and docking studies, Manvilla *et al.* [206] proposed that E3330 binds to APE1 in a pocket of the DNA-binding cleft adjacent to the catalytic site responsible for the endonuclease activity. This evidence indicates that E3330 could act as an allosteric inhibitor suppressing the conformational change of APE1 required for its redox activity, disrupting the binding and activation of transcription factors or hindering the redox chaperone activity of APE1 [206]. Manvilla *et al.* [206] also showed that E3330 may not selectively inhibit the redox function of APE1 in cells. However, inhibition of APE1 endonuclease activity was evaluated only for a high concentration of E3330 (100 µM)

[206]. In the presence of contradictory information about the inhibition mechanism of APE1 redox activity Zhang *et al.* [201] adopted hydrogen-deuterium exchange (HDX) mass spectrometry to provide novel insights into the APE1-E3330 interaction. Two sites of interaction for E3330 were identified close to the DNA repair active site [201]. Nevertheless, these interactions are probably established by the favorable electrostatic interactions between the carboxylate group of E3330 and the positively charged residues of APE1 DNA-binding site while its effectiveness as redox inhibitor is attributed to the quinone group [201]. E3330 revealed a poor inhibitory effect of the endonuclease activity *in vitro* which was observed only for concentrations higher than 100 µM [201]. A destabilization of APE1 structure was also detected during the interaction with E3330, supporting the findings of Su *et al.* [194] and highlighting the requirement of the compound binding to a partially unfolded state of the enzyme to inhibit its redox function [201].

E3330 has been shown to decrease not only the DNA-binding activity of NF-ƙB [186,199,201,207,202,208] but also of the transcription factors AP-1 [201,207,209], HIF-α [207,208,210] and STAT3 [190]. Therefore, the therapeutic potential of E3330 is associated to the targeting of different components of tumour progression. Several preclinical studies in diverse cellular models highlighted the role of E3330 in the modulation of a) cell differentiation and cellular growth [190,207,210,211], b) tumour microenvironment (inflammatory responses) [198,209,212], c) migration [210,213] and d) angiogenesis [213–215]. Furthermore, in a pancreatic tumour xenograft model E3330 induced a decrease of tumour growth rate and promising pharmacokinetic and pharmacodynamic properties [207].

E3330 was used as the scaffold for the development of novel and more potent quinone analogues able to inhibit the APE1 redox function. The naphthoquinone analogues were more potent than the lead compound and the benzoquinone analogues [203]. The most potent naphthoquinones RN7-60, RN8-51 and RN10-52 (Fig. 1.10) presented a 10-fold lower *in vitro* IC_{50} value for the APE1 redox activity when compared with the IC_{50} of E3330 [202]. They also blocked more efficiently the activation of NF-ƙB in ovarian cancer cell lines than E3330 [202]. Although the promising effects, compounds RN10-52 and RN7-60 showed to be highly reactive and to induce apoptosis at higher levels being more prone to the development of toxic side effects [202].

E3330-amide (Fig. 1.10), the methoxy amide derivative of E3330 is expected to have an improved solubility than the parent compound. Moreover, E3330-amide was a more effective inhibitor of AP-1 DNA-binding mediated by the APE1 redox activity with an

IC_{50} value of 8.5 µM while E3330 presented an IC_{50} value of 20 µM [201]. E3330-amide had also a more pronounced effect in the blockage of the growth of SKOV-3X ovarian cancer cell line [201].

The compound PNRI-299 (Fig. 1.10) was identified through a combinatorial library approach using a template designed to act as a reversible inhibitor of redox proteins [204]. PNRI-299 selectively inhibited AP-1 transcription and affinity-labeled studies showed that APE1 is the target of PNRI-299 [204].

The naturally occurring compounds resveratrol and soy isoflavones had also been proposed as redox inhibitors of APE1 [205,216]. Resveratrol (Fig. 1.10) was identified using a structure-based approach and docking studies [205]. This compound interfered with APE1-activated AP-1 DNA binding and APE1 endonuclease activity [205]. However, these findings were not supported by other authors and resveratrol is probably a non-selective inhibitor [72,217]. Similarly, the inhibition of APE1 redox activity by soy isoflavones was not corroborated by other authors [72,217]. Soy isoflavones were implied in the decrease of APE1 levels in prostate cancer cell lines which was correlated with a reduced DNA-binding of NF-ƙB [216].

1.6. APE1 IN CANCER

According to the estimated demographic features of worldwide population, the global cancer burden and cancer-related mortality is expected to increase substantially over the next years. In 2030, global cancer burden is predicted to achieve 20.3 million new cancer cases and 13.2 million cancer-related deaths compared to the 14.1 million new cancer cases and 8.2 million cancer-related deaths forecasted for 2012 and presented in the GLOBOCAN series of the International Agency for Research on Cancer [218,219].

Several lines of evidence support APE1 as a prospective target for personalised medicine in oncology. In fact, the multiplicity of functions, the correlation of alterations in the expression and subcellular distribution of APE1 with the prognosis of several types of cancer and preclinical studies suggesting that the knockdown or impairment of APE1 endonuclease activity sensitises cancer cells to chemo- and radiotherapy, granted the rationale for targeting APE1 in cancer therapy and highlighted a potential role as biomarker [16,71,73,130].

In general, the expression and intracellular localization pattern of APE1 is heterogeneous and tissue-specific [89,115,220]. In non-tumour cells, APE1 subcellular

localization is predominantly nuclear while in cell types with a high metabolic and proliferative rates APE1 can be located in cytoplasm [115,220]. Although APE1 cellular compartmentalization is not fully clarified, nuclear localization has been mainly assigned to its roles in DNA repair and to the requirement of APE1 for DNA-binding of transcription factors [115,220]. On the other hand, APE1 identification in cytoplasm can be related to extra-nuclear functions of the protein including the mitochondrial BER, its association with the endoplasmic reticulum membranes, the redox activity required to maintain recently synthesised transcription factors in a reduced state during their translocation to the nucleus and APE1 accumulation as a consequence of cellular inability to perform its degradation [98,115,220–222].

In human tumours the APE1 subcellular distribution pattern is usually disrupted when compared with the corresponding non-tumoural tissue. In tumours APE1 has shown a nuclear, cytoplasmic or nuclear/cytoplasmic localization (reviewed in [89]). These alterations have been correlated with tumour aggressiveness and prognosis in different types of cancer including colorectal [223], hepatocellular [224], ovarian [225–229], gastro-oesophageal [226], pancreatico-biliary [226,230], head and neck [231], non-small cell lung cancer (NSCLC) [232,233] and breast cancer [234–236]. The pattern of APE1 subcellular localization was also evaluated in platinum-based neoadjuvant-treated patients with gastro-oesophageal cancer [226]. In the platinum-treated group, nuclear APE1 expression was associated with a lower overall survival and cytoplasmic APE1 distribution with tumour differentiation [226].

In addition to APE1 subcellular dysregulation, APE1 levels in tumour tissue and/or in other biological samples can be important prognostic and predictive biomarkers of cancer progression and tumour sensitivity to anticancer agents [89]. Alterations in APE1 expression were reported in prostate [237], osteosarcoma [238], ovarian [229], non-small cell lung [233,239], bladder [240], hepatocellular [224], pancreatic [230], gastric [241], germ cell [242] and breast [235] cancer. Koukourakis *et al.* [231] reported that a high nuclear expression of APE1 in head and neck cancer was associated with a poor response to chemoradiotherapy. Similarly, APE1 overexpression was also associated with the resistance of NSCLC to cisplatin-based chemotherapy and with a protective effect against bleomycin and IR in germ cell tumour cell lines [233,242]. Interestingly, Sak *et al.* [240] found an improved patient survival in muscle-invasive bladder cancer following radical radiotherapy in tumours with high protein expression levels of APE1 and XRCC1. The authors suggested that this unexpected finding could be related to the tumour grade since the majority of patients enrolled in the study presented poorly differentiated tumours [240]. Schena *et al.* [239] also evaluated the

APE1 gene expression levels in blood of patients with NSCLC or squamous cell carcinoma of the head and neck (SCCHN). A positive correlation between APE1 gene level in blood and SCCHN tissue was found suggesting the potential to be used in the clinical setting as a possible blood based gene expression prognostic and predictive biomarker [239]. APE1 protein was also elevated in the serum of bladder cancer patients being a possible serological biomarker in this type of cancer [243].

In functional preclinical studies, the downregulation of APE1 with small-interfering RNAs (siRNAs) or antisense oligonucleotides sensitised several mammalian cancer cell lines to MMS [244,245], hydrogen peroxide (H_2O_2) [245,246] and to numerous therapeutic DNA-damaging agents, namely TMZ [244,247,248], BCNU [244,248], gemcitabine [249], IR [250–252] and bleomycin [251]. Moreover, a dominant-negative form of APE1 lacking the endonuclease activity enhanced the cellular sensitivity to MMS, H_2O_2, 5-FU, 5-fluorodeoxyuridine, streptozocin, TMZ and BCNU [253,254].

Several polymorphic variants have been described in the repair domain of APE1 gene as well as their possible influence in cancer susceptibility and treatment sensitivity. The APE1 polymorphisms include Asp148Glu, Gln51His, Ile64Val, Leu104Arg, Glu126Asp, Arg237Ala, Asp283Gly, Gly306Ala, Gly241Arg and Thr141Gly [130,255,256]. The functional characterization revealed that the variants Leu104Arg, Glu126Asp, Arg237Ala and Asp283Gly had a reduced endonuclease activity [256]. Although the lack of effect of Asp148Glu polymorphism in APE1 endonuclease or DNA binding activity, this polymorphic variant has been the most studied and it was associated with an increased risk of lung and gastric cancer [130,256–258].

Overall, these findings support the key value of APE1 as a predictive and prognostic biomarker as well as a promising pharmacological target in cancer therapy.

1.7. APE1 IN BREAST CANCER: ROLE AND THERAPEUTIC OPPORTUNITIES

Breast cancer is the second most frequently diagnosed neoplasm and it is the fifth cause of death from cancer in the world [219]. Although the high incidence rate of breast cancer, mortality has been decreasing, especially in the high-income countries, as a result of the advances in early diagnosis and the improvements in adjuvant therapy [219,259]. Nevertheless, breast cancer is the leading cause of cancer-related deaths in women due to the development of metastatic disease [219,259,260]. Actually, 5% to 10% of breast cancers are metastatic at diagnosis with only one-fifth of these cases surviving for 5 years [261].

The guidelines for metastatic breast cancer treatment typically include therapeutic combinations of chemotherapy, radiotherapy, surgical resection and endocrine therapy [261,262]. Chemotherapeutic drugs are frequently recommended as adjuvant systemic therapy in several types of primary breast tumours and in neoadjuvant treatment of patients with advanced disease remaining the leading treatment for triple-negative advanced breast cancer [261–263]. Despite the selection of the most appropriated therapeutic agent(s), treatment schedule and duration should be individualised. Anthracyclines as single agent or in combination regimens are usually established as first-line therapy while taxanes are the second-line drugs in triple-negative advanced breast cancer [261,262]. Moreover, taxane-based regimens can be also selected as first-line therapy since they are the standard alternative treatment for patients with refractory disease to adjuvant anthracycline-based chemotherapeutic regimens [261,262].

However, the intertumoural and intratumoural heterogeneity of primary breast tumours and the heterogeneous metastatic pattern have been commonly associated to therapeutic failure and, subsequently, to poor outcomes in the treatment of advanced breast cancer [260,264,265]. Thus, the development of targeted therapies is an attractive field to improve the overall survival in advanced breast cancer.

Based on the current available chemotherapeutic drugs which act primarily by induction of DNA damage (e.g. anthracyclines) in cancer cells, the possibility of modulating the DNA repair pathways is an emerging approach to allow the enhancement of the efficacy of a given agent. Moreover, the presence of abnormalities in genes affecting the DNA repair pathways (e.g. *BRCA1/2*, *PTEN*) is frequently observed in breast cancers rendering an alternative DNA repair pathway as an appealing target to potentiate cancer cell killing while possessing tolerable side effects [266]. In this context, APE1, an upstream protein of BER, is a rationale target for DNA repair-directed therapies in cancer treatment. Furthermore, APE1 redox function may also be involved in molecular and cellular processes required for cancer progression and metastases development, namely cell adhesion, migration, invasion and angiogenesis [72]. Therefore, the combination of an APE1 redox inhibitor with taxanes (e.g. docetaxel) might also be a novel therapeutic alternative to advanced breast cancer (Fig. 1.11).

In addition to the aforementioned differential APE1 subcellular distribution observed in breast tumours [234–236], several evidences support APE1 as a potential druggable target in breast cancer. APE1 levels were highly elevated in malignant breast tumours

relatively to non-tumoural breast tissue [235,267]. In a cohort of triple negative breast cancer (TNBC), APE1 was found to be overexpressed when compared to non-tumoural breast tissue [268]. Moreover, a dysregulation of the acetylation of APE1 was also observed by the detection of a heterogeneous staining pattern among breast cancer tissue. Since acetylated APE1 was previously linked to the modulation of APE1 endonuclease activity, concurrently with total protein overexpression, this post-translational modification may constitute an additional marker for breast cancer aggressiveness [268].

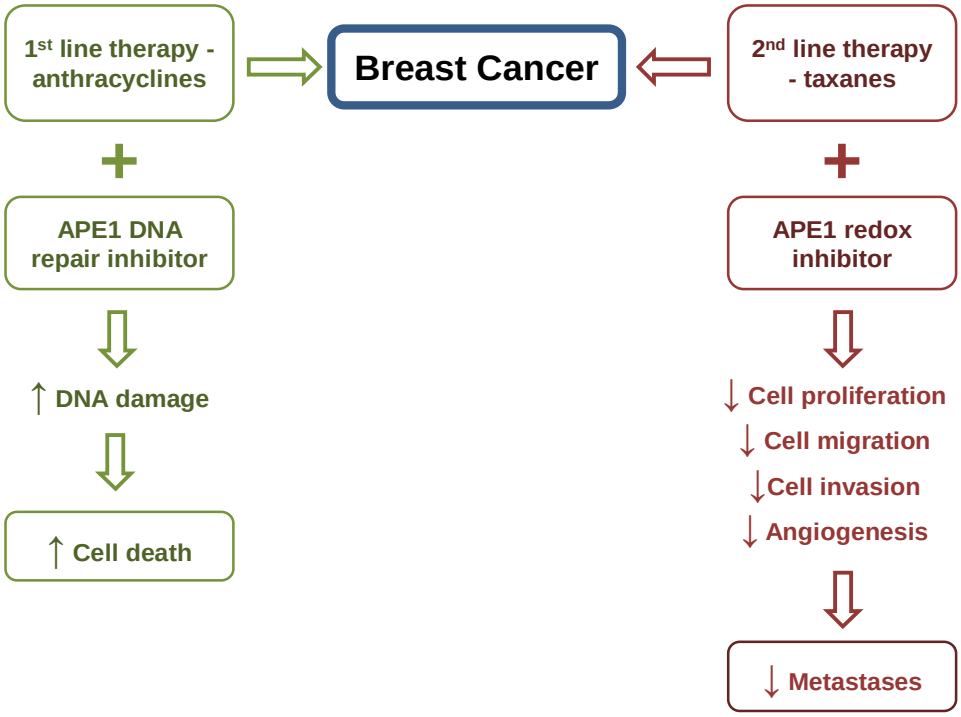


Figure 1.11 Rationale for the combination of standard chemotherapeutic drugs used in the advanced breast cancer treatment with APE1 inhibitors [2,72,261,262].

Genetic variations in APE1 may contribute to breast cancer risk and to the susceptibility to anticancer agents. Although some studies were not conclusive about the relation of APE1 variants and breast cancer risk [269,270], in a Caucasian population, the carriers of the genotype *APE1 148DD* (rs3136820) were associated with an increased risk of breast cancer [271]. The APE1 single-nucleotide polymorphism –656 T>G had also a significant role on the incidence and progression of breast cancer in a case-control study in a Chinese population [272]. The variant *APE1* 148Glu was correlated with a decreased risk of acute skin reactions after radiotherapy in breast cancer patients with normal weight [273].

Therefore, the development of APE1 inhibitors should be an important research area in the field of personalised medicine to improve the treatment of advanced breast cancer.

1.8. REFERENCES

1. Aziz K, Nowsheen S, Pantelias G, Iliakis G, Gorgoulis VG, Georgakilas AG. Targeting DNA damage and repair: embracing the pharmacological era for successful cancer therapy. Pharmacol Ther. 2012;133: 334–350.
2. Jackson SP, Bartek J. The DNA-damage response in human biology and disease. Nature. 2009;461: 1071–1078.
3. Hakem R. DNA-damage repair; the good, the bad, and the ugly. EMBO J. 2008;27: 589–605.
4. Harper JW, Elledge SJ. The DNA damage response: ten years after. Mol Cell. 2007;28: 739–745.
5. Roos WP, Kaina B. DNA damage-induced cell death: from specific DNA lesions to the DNA damage response and apoptosis. Cancer Lett. 2013;332: 237–248.
6. Hoeijmakers JHJ. DNA damage, aging, and cancer. N Engl J Med. 2009;361: 1475–1485.
7. Davis AJ, Chen DJ. DNA double strand break repair via non-homologous end-joining. Transl Cancer Res. NIH Public Access; 2013;2: 130–143.
8. Helleday T, Petermann E, Lundin C, Hodgson B, Sharma RA. DNA repair pathways as targets for cancer therapy. Nat Rev Cancer. 2008;8: 193–204.
9. Krokan HE, Bjørås M. Base excision repair. Cold Spring Harb Perspect Biol. 2013;5: a012583.
10. Kim YJ, Wilson DM 3rd. Overview of base excision repair biochemistry. Curr Mol Pharmacol. 2012;5: 3–13.
11. Krejci L, Altmannova V, Spirek M, Zhao X. Homologous recombination and its regulation. Nucleic Acids Res. 2012;40: 5795–5818.
12. Guillotin D, Martin SA. Exploiting DNA mismatch repair deficiency as a therapeutic strategy. Exp Cell Res. 2014;329: 110–115.
13. Marteijn JA, Lans H, Vermeulen W, Hoeijmakers JHJ. Understanding nucleotide excision repair and its roles in cancer and ageing. Nat Rev Mol Cell Biol. 2014;15: 465–481.
14. Eker APM, Quayle C, Chaves I, van der Horst GTJ. DNA repair in mammalian cells: Direct DNA damage reversal: elegant solutions for nasty problems. Cell Mol Life Sci. 2009;66: 968–980.
15. Yi C, He C. DNA repair by reversal of DNA damage. Cold Spring Harb Perspect Biol. 2013;5: a012575.
16. Rodrigues AS, Gomes BC, Martins C, Gromicho M, Oliveira NG, Guerreiro PS, et al. DNA repair and resistance to cancer therapy. In: Chen C, editor. New Research Directions in DNA Repair. InTech; 2013. pp. 489–528.
17. de Laat WL, Jaspers NG, Hoeijmakers JH. Molecular mechanism of nucleotide excision repair. Genes Dev. 1999;13: 768–785.
18. Jiricny J. The multifaceted mismatch-repair system. Nat Rev Mol Cell Biol. 2006;7: 335–346.

19. Lehmann AR. Translesion synthesis in mammalian cells. Exp Cell Res. 2006;312: 2673–2676.

20. Chapman JR, Taylor MRG, Boulton SJ. Playing the end game: DNA double-strand break repair pathway choice. Mol Cell. 2012;47: 497–510.

21. Jasin M, Rothstein R. Repair of strand breaks by homologous recombination. Cold Spring Harb Perspect Biol. 2013;5: a012740.

22. Walden H, Deans AJ. The Fanconi anemia DNA repair pathway: structural and functional insights into a complex disorder. Annu Rev Biophys. 2014;43: 257–278.

23. Bernstein C, Prasad AR, Nfonsam V, Bernstein H. DNA damage, DNA repair and cancer. In: Chen C, editor. New Research Directions in DNA Repair. InTech; 2013. pp. 413–465.

24. Halazonetis TD, Gorgoulis VG, Bartek J. An oncogene-induced DNA damage model for cancer development. Science. 2008;319: 1352–1355.

25. Ford D, Easton DF, Bishop DT, Narod SA, Goldgar DE. Risks of cancer in BRCA1-mutation carriers. Lancet. 1994;343: 692–695.

26. Venkitaraman AR. Cancer susceptibility and the functions of BRCA1 and BRCA2. Cell. 2002;108: 171–182.

27. Roy R, Chun J, Powell SN. BRCA1 and BRCA2: different roles in a common pathway of genome protection. Nat Rev Cancer. 2012;12: 68–78.

28. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144: 646–674.

29. Goldstein M, Kastan MB. The DNA damage response: implications for tumor responses to radiation and chemotherapy. Annu Rev Med. 2015;66: 129–143.

30. O'Grady S, Finn SP, Cuffe S, Richard DJ, O'Byrne KJ, Barr MP. The role of DNA repair pathways in cisplatin resistant lung cancer. Cancer Treat Rev. 2014;40: 1161–1170.

31. Frosina G. DNA repair and resistance of gliomas to chemotherapy and radiotherapy. Mol Cancer Res. 2009;7: 989–999.

32. Curtin NJ. DNA repair dysregulation from cancer driver to therapeutic target. Nat Rev Cancer. 2012;12: 801–817.

33. Kaelin WG. The concept of synthetic lethality in the context of anticancer therapy. Nat Rev Cancer. 2005;5: 689–698.

34. Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, *et al*. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature. 2005;434: 913–917.

35. Farmer H, McCabe N, Lord CJ, Tutt ANJ, Johnson DA, Richardson TB, *et al*. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature. 2005;434: 917–921.

36. Lindahl T. An N-glycosidase from *Escherichia coli* that releases free uracil from DNA containing deaminated cytosine residues. Proc Natl Acad Sci U S A. 1974;71: 3649–3653.

37. Lindahl T. DNA glycosylases, endonucleases for apurinic/apyrimidinic sites, and base excision-repair. Prog Nucleic Acid Res Mol Biol. 1979;22: 135–192.

38. Wilson DM 3rd, Bohr VA. The mechanics of base excision repair, and its relationship to aging and disease. DNA Repair (Amst). 2007;6: 544–559.

39. Hegde ML, Hazra TK, Mitra S. Early steps in the DNA base excision/single-strand interruption repair pathway in mammalian cells. Cell Res. 2008;18: 27–47.

40. Illuzzi JL, Wilson DM 3rd. Base excision repair: contribution to tumorigenesis and target in anticancer treatment paradigms. Curr Med Chem. 2012;19: 3922–3936.

41. Brooks SC, Adhikary S, Rubinson EH, Eichman BF. Recent advances in the structural mechanisms of DNA glycosylases. Biochim Biophys Acta. 2013;1834: 247–271.

42. Nemec AA, Wallace SS, Sweasy JB. Variant base excision repair proteins: contributors to genomic instability. Semin Cancer Biol. 2010;20: 320–328.

43. Wiederhold L, Leppard JB, Kedar P, Karimi-Busheri F, Rasouli-Nia A, Weinfeld M, *et al*. AP endonuclease-independent DNA base excision repair in human cells. Mol Cell. 2004;15: 209–220.

44. Das A, Wiederhold L, Leppard JB, Kedar P, Prasad R, Wang H, *et al*. NEIL2-initiated, APE-independent repair of oxidized bases in DNA: evidence for a repair complex in human cells. DNA Repair (Amst). 2006;5: 1439–1448.

45. Sung J-S, DeMott MS, Demple B. Long-patch base excision DNA repair of 2-deoxyribonolactone prevents the formation of DNA-protein cross-links with DNA polymerase beta. J Biol Chem. 2005;280: 39095–39103.

46. Horton JK, Prasad R, Hou E, Wilson SH. Protection against methylation-induced cytotoxicity by DNA polymerase beta-dependent long patch base excision repair. J Biol Chem. 2000;275: 2211–2218.

47. Podlutsky AJ, Dianova II, Podust VN, Bohr VA, Dianov GL. Human DNA polymerase beta initiates DNA synthesis during long-patch repair of reduced AP sites in DNA. EMBO J. 2001;20: 1477–1482.

48. Gary R, Kim K, Cornelius HL, Park MS, Matsumoto Y. Proliferating cell nuclear antigen facilitates excision in long-patch base excision repair. J Biol Chem. 1999;274: 4354–4363.

49. Kim K, Biade S, Matsumoto Y. Involvement of flap endonuclease 1 in base excision DNA repair. J Biol Chem. 1998;273: 8842–8848.

50. Fortini P, Pascucci B, Parlanti E, Sobol RW, Wilson SH, Dogliotti E. Different DNA polymerases are involved in the short- and long-patch base excision repair in mammalian cells. Biochemistry. 1998;37: 3575–3580.

51. Fortini P, Parlanti E, Sidorkina OM, Laval J, Dogliotti E. The type of DNA glycosylase determines the base excision repair pathway in mammalian cells. J Biol Chem. 1999;274: 15230–15236.

52. Fortini P, Dogliotti E. Base damage and single-strand break repair: mechanisms and functional significance of short- and long-patch repair subpathways. DNA Repair (Amst). 2007;6: 398–409.

53. Petermann E, Ziegler M, Oei SL. ATP-dependent selection between single nucleotide and long patch base excision repair. DNA Repair (Amst). 2003;2: 1101–1114.

54. Petermann E, Keil C, Oei SL. Roles of DNA ligase III and XRCC1 in regulating the switch between short patch and long patch BER. DNA Repair (Amst). 2006;5: 544–555.

55. Caldecott KW, McKeown CK, Tucker JD, Ljungquist S, Thompson LH. An interaction between the mammalian DNA repair protein XRCC1 and DNA ligase III. Mol Cell Biol. 1994;14: 68–76.

56. Hanssen-Bauer A, Solvang-Garten K, Sundheim O, Peña-Diaz J, Andersen S, Slupphaug G, *et al*. XRCC1 coordinates disparate responses and multiprotein repair complexes depending on the nature and context of the DNA damage. Environ Mol Mutagen. 2011;52: 623–635.

57. Fan J, Otterlei M, Wong H-K, Tomkinson AE, Wilson DM 3rd. XRCC1 co-localizes and physically interacts with PCNA. Nucleic Acids Res. 2004;32: 2193–2201.

58. Vidal AE, Boiteux S, Hickson ID, Radicella JP. XRCC1 coordinates the initial and late stages of DNA abasic site repair through protein-protein interactions. EMBO J. 2001;20: 6530–6539.

59. Whitehouse CJ, Taylor RM, Thistlethwaite A, Zhang H, Karimi-Busheri F, Lasko DD, *et al*. XRCC1 stimulates human polynucleotide kinase activity at damaged DNA termini and accelerates DNA single-strand break repair. Cell. 2001;104: 107–117.

60. Mani RS, Fanta M, Karimi-Busheri F, Silver E, Virgen CA, Caldecott KW, *et al*. XRCC1 stimulates polynucleotide kinase by enhancing its damage discrimination and displacement from DNA repair intermediates. J Biol Chem. 2007;282: 28004–28013.

61. Kubota Y, Nash RA, Klungland A, Schär P, Barnes DE, Lindahl T. Reconstitution of DNA base excision-repair with purified human proteins: interaction between DNA polymerase beta and the XRCC1 protein. EMBO J. 1996;15: 6662–6670.

62. Dianova II, Sleeth KM, Allinson SL, Parsons JL, Breslin C, Caldecott KW, *et al*. XRCC1-DNA polymerase beta interaction is required for efficient base excision repair. Nucleic Acids Res. 2004;32: 2550–2555.

63. Caldecott KW, Aoufouchi S, Johnson P, Shall S. XRCC1 polypeptide interacts with DNA polymerase beta and possibly poly(ADP-ribose) polymerase, and DNA ligase III is a novel molecular “nick-sensor” *in vitro*. Nucleic Acids Res. 1996;24: 4387–4394.

64. Campalans A, Marsin S, Nakabeppu Y, O’connor TR, Boiteux S, Radicella JP. XRCC1 interactions with multiple DNA glycosylases: a model for its recruitment to base excision repair. DNA Repair (Amst). 2005;4: 826–835.

65. Schreiber V, Amé J-C, Dollé P, Schultz I, Rinaldi B, Fraulob V, et al. Poly(ADP-ribose) polymerase-2 (PARP-2) is required for efficient base excision DNA repair in association with PARP-1 and XRCC1. J Biol Chem. 2002;277: 23028–23036.

66. El-Khamisy SF, Masutani M, Suzuki H, Caldecott KW. A requirement for PARP-1 for the assembly or stability of XRCC1 nuclear foci at sites of oxidative DNA damage. Nucleic Acids Res. 2003;31: 5526–5533.

67. Amé J-C, Spenlehauer C, de Murcia G. The PARP superfamily. Bioessays. 2004;26: 882–893.

68. Kim MY, Zhang T, Kraus WL. Poly(ADP-ribosyl)ation by PARP-1: “PAR-laying” NAD+ into a nuclear signal. Genes Dev. 2005;19: 1951–1967.

69. Grösch S, Fritz G, Kaina B. Apurinic endonuclease (Ref-1) is induced in mammalian cells by oxidative stress and involved in clastogenic adaptation. Cancer Res. 1998;58: 4410–4416.

70. Fung H, Demple B. A vital role for Ape1/Ref1 protein in repairing spontaneous DNA damage in human cells. Mol Cell. 2005;17: 463–470.

71. Tell G, Fantini D, Quadrifoglio F. Understanding different functions of mammalian AP endonuclease (APE1) as a promising tool for cancer treatment. Cell Mol Life Sci. 2010;67: 3589–3608.

72. Kelley MR, Georgiadis MM, Fishel ML. APE1/Ref-1 role in redox signaling: translational applications of targeting the redox function of the DNA repair/redox protein APE1/Ref-1. Curr Mol Pharmacol. 2012;5: 36–53.

73. Bapat A, Fishel ML, Kelley MR. Going ape as an approach to cancer therapeutics. Antioxid Redox Signal. 2009;11: 651–668.

74. Doetsch PW, Cunningham RP. The enzymology of apurinic/apyrimidinic endonucleases. Mutat Res. 236: 173–201.

75. Robson CN, Hickson ID. Isolation of cDNA clones encoding a human apurinic/apyrimidinic endonuclease that corrects DNA repair and mutagenesis defects in *E. coli xth* (exonuclease III) mutants. Nucleic Acids Res. 1991;19: 5519–2553.

76. Demple B, Herman T, Chen DS. Cloning and expression of APE, the cDNA encoding the major human apurinic endonuclease: definition of a family of DNA repair enzymes. Proc Natl Acad Sci U S A. 1991;88: 11450–11454.

77. Gorman MA, Morera S, Rothwell DG, de La Fortelle E, Mol CD, Tainer JA, *et al*. The crystal structure of the human DNA repair endonuclease HAP1 suggests the recognition of extra-helical deoxyribose at DNA abasic sites. EMBO J. 1997;16: 6548–6558.

78. Strauss PR, Holt CM. Domain mapping of human apurinic/apyrimidinic endonuclease. Structural and functional evidence for a disordered amino terminus and a tight globular carboxyl domain. J Biol Chem. 1998;273: 14435–14441.

79. Mol CD, Izumi T, Mitra S, Tainer JA. DNA-bound structures and mutants reveal abasic DNA binding by APE1 and DNA repair coordination. Nature. 2000;403: 451–456.

80. Georgiadis MM, Luo M, Gaur RK, Delaplane S, Li X, Kelley MR. Evolution of the redox function in mammalian apurinic/apyrimidinic endonuclease. Mutat Res. 2008;643: 54–63.

81. Tell G, Quadrifoglio F, Tiribelli C, Kelley MR. The many functions of APE1/Ref-1: not only a DNA repair enzyme. Antioxid Redox Signal. 2009;11: 601–620.

82. Fantini D, Vascotto C, Marasco D, D'Ambrosio C, Romanello M, Vitagliano L, *et al*. Critical lysine residues within the overlooked N-terminal domain of human APE1 regulate its biological functions. Nucleic Acids Res. 2010;38: 8239–8256.

83. Xanthoudakis S, Miao GG, Curran T. The redox and DNA-repair activities of Ref-1 are encoded by nonoverlapping domains. Proc Natl Acad Sci U S A. 1994;91: 23–27.

84. Jackson EB, Theriot CA, Chattopadhyay R, Mitra S, Izumi T. Analysis of nuclear transport signals in the human apurinic/apyrimidinic endonuclease (APE1/Ref1). Nucleic Acids Res. 2005;33: 3303–3312.

85. Al-Safi RI, Odde S, Shabaik Y, Neamati N. Small-molecule inhibitors of APE1 DNA repair function: an overview. Curr Mol Pharmacol. 2012;5: 14–35.

86. Gates KS. An overview of chemical processes that damage cellular DNA: spontaneous hydrolysis, alkylation, and reactions with radicals. Chem Res Toxicol. 2009;22: 1747–1760.

87. Wilson DM 3rd, Barsky D. The major human abasic endonuclease: formation, consequences and repair of abasic lesions in DNA. Mutat Res. 2001;485: 283–307.

88. Lindahl T. Instability and decay of the primary structure of DNA. Nature. 1993;362: 709–715.

89. Abbotts R, Madhusudan S. Human AP endonuclease 1 (APE1): from mechanistic insights to druggable target in cancer. Cancer Treat Rev. 2010;36: 425–435.

90. Boiteux S, Guillet M. Abasic sites in DNA: repair and biological consequences in Saccharomyces cerevisiae. DNA Repair (Amst). 2004;3: 1–12.

91. Pourquier P, Ueng LM, Kohlhagen G, Mazumder A, Gupta M, Kohn KW, *et al*. Effects of uracil incorporation, DNA mismatches, and abasic sites on cleavage and religation activities of mammalian topoisomerase I. J Biol Chem. 1997;272: 7792–7796.

92. Parsons JL, Dianova II, Dianov GL. APE1 is the major 3'-phosphoglycolate activity in human cell extracts. Nucleic Acids Res. 2004;32: 3531–3536.

93. Wilson DM 3rd. Properties of and substrate determinants for the exonuclease activity of human apurinic endonuclease Ape1. J Mol Biol. 2003;330: 1027–1037.

94. Chou K-M, Cheng Y-C. An exonucleolytic activity of human apurinic/apyrimidinic endonuclease on 3' mispaired DNA. Nature. 2002;415: 655–659.

95. Chou KM, Kukhanova M, Cheng YC. A novel action of human apurinic/apyrimidinic endonuclease: excision of L-configuration deoxyribonucleoside analogs from the 3' termini of DNA. J Biol Chem. 2000;275: 31009–31015.

96. Chou K-M, Cheng Y-C. The exonuclease activity of human apurinic/apyrimidinic endonuclease (APE1). Biochemical properties and inhibition by the natural dinucleotide Gp4G. J Biol Chem. 2003;278: 18289–18296.

97. Gros L, Ishchenko AA, Ide H, Elder RH, Saparbaev MK. The major human AP endonuclease (Ape1) is involved in the nucleotide incision repair pathway. Nucleic Acids Res. 2004;32: 73–81.

98. Kazak L, Reyes A, Holt IJ. Minimizing the damage: repair pathways keep mitochondrial DNA intact. Nat Rev Mol Cell Biol. 2012;13: 659–671.

99. Tell G, Crivellato E, Pines A, Paron I, Pucillo C, Manzini G, *et al*. Mitochondrial localization of APE/Ref-1 in thyroid cells. Mutat Res. 2001;485: 143–152.

100. Li M, Zhong Z, Zhu J, Xiang D, Dai N, Cao X, *et al*. Identification and characterization of mitochondrial targeting sequence of human apurinic/apyrimidinic endonuclease 1. J Biol Chem. 2010;285: 14871–1481.

101. Angkeow P, Deshpande SS, Qi B, Liu Y-X, Park YC, Jeon BH, *et al*. Redox factor-1: an extra-nuclear role in the regulation of endothelial oxidative stress and apoptosis. Cell Death Differ. 2002;9: 717–725.

102. den Hartog G, Chattopadhyay R, Ablack A, Hall EH, Butcher LD, Bhattacharyya A, *et al*. Regulation of Rac1 and reactive oxygen species production in response to infection of gastrointestinal epithelia. PLoS Pathog. 2016;12: e1005382.

103. Barzilay G, Walker LJ, Robson CN, Hickson ID. Site-directed mutagenesis of the human DNA repair enzyme HAP1: identification of residues important for AP endonuclease and RNase H activity. Nucleic Acids Res. 1995;23: 1544–1550.

104. Kim SE, Gorrell A, Rader SD, Lee CH. Endoribonuclease activity of human apurinic/apyrimidinic endonuclease 1 revealed by a real-time fluorometric assay. Anal Biochem. 2010;398: 69–75.

105. Barnes T, Kim WC, Mantha AK, Kim SE, Izumi T, Mitra S, *et al*. Identification of Apurinic/apyrimidinic endonuclease 1 (APE1) as the endoribonuclease that cleaves c-*myc* mRNA. Nucleic Acids Res. 2009;37: 3946–3958.

106. Vascotto C, Fantini D, Romanello M, Cesaratto L, Deganuto M, Leonardi A, *et al*. APE1/Ref-1 interacts with NPM1 within nucleoli and plays a role in the rRNA quality control process. Mol Cell Biol. 2009;29: 1834–1854.

107. Kim WC, Berquist BR, Chohan M, Uy C, Wilson DM 3rd, Lee CH. Characterization of the endoribonuclease active site of human apurinic/apyrimidinic endonuclease 1. J Mol Biol. 2011;411: 960–971.

108. Berquist BR, McNeill DR, Wilson DM 3rd. Characterization of abasic endonuclease activity of human Ape1 on alternative substrates, as well as effects of ATP and sequence context on AP site incision. J Mol Biol. 2008;379: 17–27.

109. Vascotto C, Lirussi L, Poletto M, Tiribelli M, Damiani D, Fabbro D, *et al*. Functional regulation of the apurinic/apyrimidinic endonuclease 1 by nucleophosmin: impact on tumor biology. Oncogene. 2014;33: 2876–2887.

110. Sidorenko VS, Nevinsky GA, Zharkov DO. Mechanism of interaction between human 8-oxoguanine-DNA glycosylase and AP endonuclease. DNA Repair (Amst). 2007;6: 317–328.

111. Dianova II, Bohr VA, Dianov GL. Interaction of human AP endonuclease 1 with flap endonuclease 1 and proliferating cell nuclear antigen involved in long-patch base excision repair. Biochemistry. 2001;40: 12639–12644.

112. Bennett RA, Wilson DM 3rd, Wong D, Demple B. Interaction of human apurinic endonuclease and DNA polymerase beta in the base excision repair pathway. Proc Natl Acad Sci U S A. 1997;94: 7166–7169.

113. Prasad R, Dyrkheeva N, Williams J, Wilson SH. Mammalian Base Excision Repair: functional partnership between PARP-1 and APE1 in AP-site repair. PLoS One. 2015;10: e0124269.

114. Thakur S, Dhiman M, Tell G, Mantha AK. A review on protein-protein interaction network of APE1/Ref-1 and its associated biological functions. Cell Biochem Funct. 2015;33: 101–112.

115. Scott TL, Rangaswamy S, Wicker CA, Izumi T. Repair of oxidative DNA damage and cancer: recent progress in DNA base excision repair. Antioxid Redox Signal. 2014;20: 708–726.

116. Zhao J, Gao F, Zhang Y, Wei K, Liu Y, Deng X. Bcl2 inhibits abasic site repair by down-regulating APE1 endonuclease activity. J Biol Chem. 2008;283: 9925–9932.

117. Vascotto C, Cesaratto L, Zeef LAH, Deganuto M, D'Ambrosio C, Scaloni A, *et al*. Genome-wide analysis and proteomic studies reveal APE1/Ref-1 multifunctional role in mammalian cells. Proteomics. 2009;9: 1058–1074.

118. Okazaki T, Chung U, Nishishita T, Ebisu S, Usuda S, Mishiro S, *et al*. A redox factor protein, ref1, is involved in negative gene regulation by extracellular calcium. J Biol Chem. 1994;269: 27855–27862.

119. Kuninger DT, Izumi T, Papaconstantinou J, Mitra S. Human AP-endonuclease 1 and hnRNP-L interact with a nCaRE-like repressor element in the AP-endonuclease 1 promoter. Nucleic Acids Res. 2002;30: 823–829.

120. Bhakat KK, Izumi T, Yang S-H, Hazra TK, Mitra S. Role of acetylated human AP-endonuclease (APE1/Ref-1) in regulation of the parathyroid hormone gene. EMBO J. 2003;22: 6299–6309.

121. Busso CS, Lake MW, Izumi T. Posttranslational modification of mammalian AP endonuclease (APE1). Cell Mol Life Sci. 2010;67: 3609–3620.

122. Fantini D, Vascotto C, Deganuto M, Bivi N, Gustincich S, Marcon G, *et al*. APE1/Ref-1 regulates PTEN expression mediated by Egr-1. Free Radic Res. 2008;42: 20–29.

123. Chattopadhyay R, Das S, Maiti AK, Boldogh I, Xie J, Hazra TK, *et al*. Regulatory role of human AP-endonuclease (APE1/Ref-1) in YB-1-mediated activation of the multidrug resistance gene MDR1. Mol Cell Biol. 2008;28: 7066–7080.

124. Sengupta S, Mantha AK, Mitra S, Bhakat KK. Human AP endonuclease (APE1/Ref-1) and its acetylation regulate YB-1-p300 recruitment and RNA polymerase II loading in the drug-induced activation of multidrug resistance gene MDR1. Oncogene. 2011;30: 482–493.

125. Yacoub A, Kelley MR, Deutsch WA. The DNA repair activity of human redox/repair protein APE/Ref-1 is inactivated by phosphorylation. Cancer Res. 1997;57: 5457–5459.

126. Fritz G, Kaina B. Phosphorylation of the DNA repair protein APE/REF-1 by CKII affects redox regulation of AP-1. Oncogene. 1999;18: 1033–1040.

127. Qu J, Liu GH, Huang B, Chen C. Nitric oxide controls nuclear export of APE1/Ref-1 through S-nitrosation of cysteines 93 and 310. Nucleic Acids Res. 2007;35: 2522–2532.

128. Kim YJ, Kim D, Illuzzi JL, Delaplane S, Su D, Bernier M, *et al*. S-glutathionylation of cysteine 99 in the APE1 protein impairs abasic endonuclease activity. J Mol Biol. 2011;414: 313–326.

129. Busso CS, Iwakuma T, Izumi T. Ubiquitination of mammalian AP endonuclease (APE1) regulated by the p53-MDM2 signaling pathway. Oncogene. 2009;28: 1616–1625.

130. Mohan V, Madhusudan S. DNA base excision repair: Evolving biomarkers for personalized therapies in cancer. In: Chen C, editor. New Research Directions in DNA Repair. InTech; 2013. pp. 529–557.

131. Fishel ML, Kelley MR. The DNA base excision repair protein Ape1/Ref-1 as a therapeutic and chemopreventive target. Mol Aspects Med. 2007;28: 375–395.

132. Sultana R, McNeill DR, Abbotts R, Mohammed MZ, Zdzienicka MZ, Qutob H, *et al*. Synthetic lethal targeting of DNA double-strand break repair deficient cells by human apurinic/apyrimidinic endonuclease inhibitors. Int J cancer. 2012;131: 2433–2444.

133. Abbotts R, Jewell R, Nsengimana J, Maloney DJ, Simeonov A, Seedhouse C, *et al*. Targeting human apurinic/apyrimidinic endonuclease 1 (APE1) in phosphatase and tensin homolog (PTEN) deficient melanoma cells for personalized therapy. Oncotarget. 2014;5: 3273–3286.

134. Beernink PT, Segelke BW, Hadi MZ, Erzberger JP, Wilson DM 3rd, Rupp B. Two divalent metal ions in the active site of a new crystal form of human apurinic/apyrimidinic endonuclease, Ape1: implications for the catalytic mechanism. J Mol Biol. 2001;307: 1023–1034.

135. Tsutakawa SE, Shin DS, Mol CD, Izumi T, Arvai AS, Mantha AK, *et al*. Conserved structural chemistry for incision activity in structurally non-homologous apurinic/apyrimidinic endonuclease APE1 and endonuclease IV DNA repair enzymes. J Biol Chem. 2013;288: 8445–8455.

136. Manvilla BA, Pozharski E, Toth EA, Drohat AC. Structure of human apurinic/apyrimidinic endonuclease 1 with the essential Mg^{2+} cofactor. Acta Crystallogr D Biol Crystallogr. 2013;69: 2555–2562.

137. He H, Chen Q, Georgiadis MM. High-resolution crystal structures reveal plasticity in the metal binding site of apurinic/apyrimidinic endonuclease I. Biochemistry. 2014;53: 6520–6529.

138. Freudenthal BD, Beard WA, Cuneo MJ, Dyrkheeva NS, Wilson SH. Capturing snapshots of APE1 processing DNA damage. Nat Struct Mol Biol. 2015;22: 924–931.

139. Barzilay G, Mol CD, Robson CN, Walker LJ, Cunningham RP, Tainer JA, *et al*. Identification of critical active-site residues in the multifunctional human DNA repair enzyme HAP1. Nat Struct Biol. 1995;2: 561–568.

140. Kane CM, Linn S. Purification and characterization of an apurinic/apyrimidinic endonuclease from HeLa cells. J Biol Chem. 1981;256: 3405–3414.

141. Oezguen N, Schein CH, Peddi SR, Power TD, Izumi T, Braun W. A “moving metal mechanism” for substrate cleavage by the DNA repair endonuclease APE-1. Proteins. 2007;68: 313–323.

142. Oezguen N, Mantha AK, Izumi T, Schein CH, Mitra S, Braun W. MD simulation and experimental evidence for Mg^{2+} binding at the B site in human AP endonuclease 1. Bioinformation. 2011;7: 184–198.

143. Lipton AS, Heck RW, Primak S, McNeill DR, Wilson DM 3rd, Ellis PD. Characterization of Mg^{2+} binding to the DNA repair protein apurinic/apyrimidic endonuclease 1 via solid-state ^{25}Mg NMR spectroscopy. J Am Chem Soc. 2008;130: 9332–9341.

144. Liuzzi M, Talpaert-Borle M. A new approach to the study of the base-excision repair pathway using methoxyamine. J Biol Chem. 1985;260: 5252–5258.

145. Yan L, Bulgar A, Miao Y, Mahajan V, Donze JR, Gerson SL, *et al*. Combined treatment with temozolomide and methoxyamine: blocking apurininc/pyrimidinic site repair coupled with targeting topoisomerase IIalpha. Clin Cancer Res. 2007;13: 1532–1539.

146. Fishel ML, He Y, Smith ML, Kelley MR. Manipulation of base excision repair to sensitize ovarian cancer cells to alkylating agent temozolomide. Clin Cancer Res. 2007;13: 260–267.

147. Liu L, Gerson SL. Therapeutic impact of methoxyamine: blocking repair of abasic sites in the base excision repair pathway. Curr Opin Investig Drugs. 2004;5: 623–627.

148. Taverna P, Liu L, Hwang HS, Hanson AJ, Kinsella TJ, Gerson SL. Methoxyamine potentiates DNA single strand breaks and double strand breaks induced by temozolomide in colon cancer cells. Mutat Res. 2001;485: 269–281.

149. Montaldi AP, Sakamoto-Hojo ET. Methoxyamine sensitizes the resistant glioblastoma T98G cell line to the alkylating agent temozolomide. Clin Exp Med. 2013;13: 279–288.

150. Liu L, Yan L, Donze JR, Gerson SL. Blockage of abasic site repair enhances antitumor efficacy of 1,3-bis-(2-chloroethyl)-1-nitrosourea in colon tumor xenografts. Mol Cancer Ther. 2003;2: 1061–1066.

151. Bulgar AD, Weeks LD, Miao Y, Yang S, Xu Y, Guo C, *et al*. Removal of uracil by uracil DNA glycosylase limits pemetrexed cytotoxicity: overriding the limit with methoxyamine to inhibit base excision repair. Cell Death Dis. 2012;3: e252.

152. Taverna P, Hwang H-S, Schupp JE, Radivoyevitch T, Session NN, Reddy G, *et al*. Inhibition of base excision repair potentiates iododeoxyuridine-induced cytotoxicity and radiosensitization. Cancer Res. 2003;63: 838–846.

153. Yan T, Seo Y, Schupp JE, Zeng X, Desai AB, Kinsella TJ. Methoxyamine potentiates iododeoxyuridine-induced radiosensitization by altering cell cycle kinetics and enhancing senescence. Mol Cancer Ther. 2006;5: 893–902.

154. Gordon MS, Rosen LS, Mendelson D, Ramanathan RK, Goldman J, Liu L, *et al*. A phase 1 study of TRC102, an inhibitor of base excision repair, and pemetrexed in patients with advanced solid tumors. Invest New Drugs. 2013;31: 714–723.

155. Caimi P, Cooper B, William BM, Campagnaro EL, Creger RJ, Afable M, *et al*. Phase I trial of the base–excision repair blocker methoxyamine (TRC-102) combined with fludarabine in relapsed/refractory chronic lymphocytic leukemia (CLL) and lymphoid malignancies. Blood. 2014;124: 4688.

156. Madhusudan S, Smart F, Shrimpton P, Parsons JL, Gardiner L, Houlbrook S, *et al*. Isolation of a small molecule inhibitor of DNA base excision repair. Nucleic Acids Res. 2005;33: 4711–4724.

157. Luo M, Kelley MR. Inhibition of the human apurinic/apyrimidinic endonuclease (APE1) repair activity and sensitization of breast cancer cells to DNA alkylating agents with lucanthone. Anticancer Res. 2004;24: 2127–2134.

158. Naidu MD, Agarwal R, Pena LA, Cunha L, Mezei M, Shen M, *et al*. Lucanthone and its derivative hycanthone inhibit apurinic endonuclease-1 (APE1) by direct protein binding. PLoS One. 2011;6: e23679.

159. Bapat A, Glass LS, Luo M, Fishel ML, Long EC, Georgiadis MM, *et al*. Novel small-molecule inhibitor of apurinic/apyrimidinic endonuclease 1 blocks proliferation and reduces viability of glioblastoma cells. J Pharmacol Exp Ther. 2010;334: 988–998.

160. Simeonov A, Kulkarni A, Dorjsuren D, Jadhav A, Shen M, McNeill DR, *et al*. Identification and characterization of inhibitors of human apurinic/apyrimidinic endonuclease APE1. PLoS One. 2009;4: e5740.

161. Dorjsuren D, Kim D, Vyjayanti VN, Maloney DJ, Jadhav A, Wilson DM 3rd, *et al*. Diverse small molecule inhibitors of human apurinic/apyrimidinic endonuclease APE1 identified from a screen of a large public collection. PLoS One. 2012;7: e47974.

162. Seiple LA, Cardellina JH, Akee R, Stivers JT. Potent inhibition of human apurinic/apyrimidinic endonuclease 1 by arylstibonic acids. Mol Pharmacol. 2008;73: 669–677.

163. Rai G, Vyjayanti VN, Dorjsuren D, Simeonov A, Jadhav A, Wilson DM 3rd, *et al*. Synthesis, biological evaluation, and structure–activity relationships of a novel class of apurinic/apyrimidinic endonuclease 1 inhibitors. J Med Chem. 2012;55: 3101–3112.

164. Aiello F, Shabaik Y, Esqueda A, Sanchez TW, Grande F, Garofalo A, *et al*. Design and synthesis of 3-carbamoylbenzoic acid derivatives as inhibitors of human apurinic/apyrimidinic endonuclease 1 (APE1). ChemMedChem. 2012;7: 1825–1839.

165. Mohammed MZ, Vyjayanti VN, Laughton CA, Dekker L V, Fischer PM, Wilson DM 3rd, *et al*. Development and evaluation of human AP endonuclease inhibitors in melanoma and glioma cell lines. Br J Cancer. 2011;104: 653–663.

166. Zawahir Z, Dayam R, Deng J, Pereira C, Neamati N. Pharmacophore guided discovery of small-molecule human apurinic/apyrimidinic endonuclease 1 inhibitors. J Med Chem. 2009;52: 20–32.

167. Srinivasan A, Wang L, Cline CJ, Xie Z, Sobol RW, Xie XQ, *et al*. Identification and characterization of human apurinic/apyrimidinic endonuclease-1 inhibitors. Biochemistry. 2012;51: 6246–6259.

168. Ruiz FM, Francis SM, Tintoré M, Ferreira R, Gil-Redondo R, Morreale A, *et al*. Receptor-based virtual screening and biological characterization of human apurinic/apyrimidinic endonuclease (Ape1) inhibitors. ChemMedChem. 2012;7: 2168–2178.

169. Feng Z, Kochanek S, Close D, Wang L, Srinivasan A, Almehizia AA, *et al*. Design and activity of AP endonuclease-1 inhibitors. J Chem Biol. 2015;8: 79–93.

170. Kotera N, Poyer F, Granzhan A, Teulade-Fichou M-P. Efficient inhibition of human AP endonuclease 1 (APE1) via substrate masking by abasic site-binding macrocyclic ligands. Chem Commun (Camb). 2015;51: 15948–15951.

171. Nicco C, Laurent A, Chereau C, Weill B, Batteux F. Differential modulation of normal and tumor cell proliferation by reactive oxygen species. Biomed Pharmacother. 2005;59: 169–174.

172. Laurent A, Nicco C, Chéreau C, Goulvestre C, Alexandre J, Alves A, *et al*. Controlling tumor growth by modulating endogenous production of reactive oxygen species. Cancer Res. 2005;65: 948–956.

173. Sosa V, Moliné T, Somoza R, Paciucci R, Kondoh H, LLeonart ME. Oxidative stress and cancer: An overview. Ageing Res Rev. 2013;12: 376–390.

174. Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat Rev Drug Discov. 2009;8: 579–591.

175. Gewirtz DA. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. Biochem Pharmacol. 1999;57: 727–741.

176. Marullo R, Werner E, Degtyareva N, Moore B, Altavilla G, Ramalingam SS, *et al*. Cisplatin induces a mitochondrial-ROS response that contributes to cytotoxicity depending on mitochondrial redox status and bioenergetic functions. PLoS One. 2013;8: e81162.

177. Azzam EI, Jay-Gerin J-P, Pain D. Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. Cancer Lett. 2012;327: 48–60.

178. Alexandre J, Batteux F, Nicco C, Chéreau C, Laurent A, Guillevin L, *et al*. Accumulation of hydrogen peroxide is an early and crucial step for paclitaxel-induced cancer cell death both in vitro and in vivo. Int J cancer. 2006;119: 41–48.

179. Mizumachi T, Suzuki S, Naito A, Carcel-Trullols J, Evans TT, Spring PM, *et al*. Increased mitochondrial DNA induces acquired docetaxel resistance in head and neck cancer cells. Oncogene. 2008;27: 831–838.

180. Wondrak GT. Redox-directed cancer therapeutics: molecular mechanisms and opportunities. Antioxid Redox Signal. 2009;11: 3013–3069.

181. Marty M, Espié M, Llombart A, Monnier A, Rapoport BL, Stahalova V. Multicenter randomized phase III study of the cardioprotective effect of dexrazoxane (Cardioxane®) in advanced/metastatic breast cancer patients treated with anthracycline-based chemotherapy. Ann Oncol. 2006;17: 614–622.

182. Konorev EA, Kennedy MC, Kalyanaraman B. Cell-permeable superoxide dismutase and glutathione peroxidase mimetics afford superior protection against doxorubicin-induced cardiotoxicity: the role of reactive oxygen and nitrogen intermediates. Arch Biochem Biophys. 1999;368: 421–428.

183. Manda G, Isvoranu G, Comanescu MV, Manea A, Debelec Butuner B, Korkmaz KS. The redox biology network in cancer pathophysiology and therapeutics. Redox Biol. 2015;5: 347–357.

184. Hornsveld MM, Dansen DTB. The hallmarks of cancer from a redox perspective. Antioxid Redox Signal. 2016;25: 300–325.

185. Ray PD, Huang BW, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cell Signal. 2012;24: 981–990.

186. Nishi T, Shimizu N, Hiramoto M, Sato I, Yamaguchi Y, Hasegawa M, *et al*. Spatial redox regulation of a critical cysteine residue of NF-kappa B in vivo. J Biol Chem. 2002;277: 44548–44556.

187. Xanthoudakis S, Curran T. Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity. EMBO J. 1992;11: 653–665.

188. Huang LE, Arany Z, Livingston DM, Bunn HF. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. J Biol Chem. 1996;271: 32253–32259.

189. Jayaraman L, Murthy KG, Zhu C, Curran T, Xanthoudakis S, Prives C. Identification of redox/repair protein Ref-1 as a potent activator of p53. Genes Dev. 1997;11: 558–570.

190. Cardoso AA, Jiang Y, Luo M, Reed AM, Shahda S, He Y, *et al*. APE1/Ref-1 regulates STAT3 transcriptional activity and APE1/Ref-1-STAT3 dual-targeting effectively inhibits pancreatic cancer cell survival. PLoS One. 2012;7: e47462.

191. Cao X, Kambe F, Ohmori S, Seo H. Oxidoreductive modification of two cysteine residues in paired domain by Ref-1 regulates DNA-binding activity of Pax-8. Biochem Biophys Res Commun. 2002;297: 288–293.

192. Walker LJ, Robson CN, Black E, Gillespie D, Hickson ID. Identification of residues in the human DNA repair enzyme HAP1 (Ref-1) that are essential for redox regulation of Jun DNA binding. Mol Cell Biol. 1993;13: 5370–5376.

193. Luo M, Zhang J, He H, Su D, Chen Q, Gross ML, *et al*. Characterization of the redox activity and disulfide bond formation in apurinic/apyrimidinic endonuclease. Biochemistry. 2012;51: 695–705.

194. Su D, Delaplane S, Luo M, Rempel DL, Vu B, Kelley MR, *et al*. Interactions of apurinic/apyrimidinic endonuclease with a redox inhibitor: evidence for an alternate conformation of the enzyme. Biochemistry. 2011;50: 82–92.

195. Hirota K, Matsui M, Iwata S, Nishiyama A, Mori K, Yodoi J. AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. Proc Natl Acad Sci U S A. 1997;94: 3633–3638.

196. Seemann S, Hainaut P. Roles of thioredoxin reductase 1 and APE/Ref-1 in the control of basal p53 stability and activity. Oncogene. 2005;24: 3853–3863.

197. Nagakawa J, Hishinuma I, Hirota K, Miyamoto K, Yamanaka T, Yamatsu I, *et al*. Protective effects of E3330, a novel quinone derivative, on galactosamine/tumor necrosis factor-α-induced hepatitis in mice. Eur J Pharmacol. 1992;229: 63–67.

198. Miyamoto K, Nagakawa J, Hishinuma I, Hirota K, Yasuda M, Yamanaka T, *et al*. Suppressive effects of E3330, a novel quinone derivative, on tumor necrosis factor-alpha generation from monocytes and macrophages. Agents Actions. 1992;37: 297–304.

199. Hiramoto M, Shimizu N, Sugimoto K, Tang J, Kawakami Y, Ito M, *et al*. Nuclear targeted suppression of NF-kappa B activity by the novel quinone derivative E3330. J Immunol. 1998;160: 810–819.

200. Shimizu N, Sugimoto K, Tang J, Nishi T, Sato I, Hiramoto M, *et al*. High-performance affinity beads for identifying drug receptors. Nat Biotechnol. 2000;18: 877–881.

201. Zhang J, Luo M, Marasco D, Logsdon D, LaFavers KA, Chen Q, *et al*. Inhibition of apurinic/apyrimidinic endonuclease I's redox activity revisited. Biochemistry. 2013;52: 2955–2966.

202. Kelley MR, Luo M, Reed A, Su D, Delaplane S, Borch RF, *et al*. Functional analysis of novel analogues of E3330 that block the redox signaling activity of the multifunctional AP endonuclease/redox signaling enzyme APE1/Ref-1. Antioxid Redox Signal. 2011;14: 1387–1401.

203. Nyland RL, Luo M, Kelley MR, Borch RF. Design and synthesis of novel quinone inhibitors targeted to the redox function of apurinic/apyrimidinic endonuclease 1/redox enhancing factor-1 (Ape1/ref-1). J Med Chem. 2010;53: 1200–1210.

204. Nguyen C, Teo J-L, Matsuda A, Eguchi M, Chi EY, Henderson WR, *et al*. Chemogenomic identification of Ref-1/AP-1 as a therapeutic target for asthma. Proc Natl Acad Sci U S A. 2003;100: 1169–1173.

205. Yang S, Irani K, Heffron SE, Jurnak F, Meyskens FL. Alterations in the expression of the apurinic/apyrimidinic endonuclease-1/redox factor-1 (APE/Ref-1) in human melanoma and identification of the therapeutic potential of resveratrol as an APE/Ref-1 inhibitor. Mol Cancer Ther. 2005;4: 1923–1935.

206. Manvilla BA, Wauchope O, Seley-Radtke KL, Drohat AC. NMR studies reveal an unexpected binding site for a redox inhibitor of AP endonuclease 1. Biochemistry. 2011;50: 10540–10549.

207. Fishel ML, Jiang Y, Rajeshkumar N V, Scandura G, Sinn AL, He Y, *et al*. Impact of APE1/Ref-1 redox inhibition on pancreatic tumor growth. Mol Cancer Ther. 2011;10: 1698–1708.

208. Zou GM, Maitra A. Small-molecule inhibitor of the AP endonuclease 1/REF-1 E3330 inhibits pancreatic cancer cell growth and migration. Mol Cancer Ther. 2008;7: 2012–2021.

209. Jedinak A, Dudhgaonkar S, Kelley MR, Sliva D. Apurinic/Apyrimidinic endonuclease 1 regulates inflammatory response in macrophages. Anticancer Res. 2011;31: 379–385.

210. Zou GM, Karikari C, Kabe Y, Handa H, Anders RA, Maitra A. The Ape-1/Ref-1 redox antagonist E3330 inhibits the growth of tumor endothelium and endothelial progenitor cells: therapeutic implications in tumor angiogenesis. J Cell Physiol. 2009;219: 209–218.

211. Jiang S, Zhu L, Tang H, Zhang M, Chen Z, Fei J, *et al*. Ape1 regulates WNT/β-catenin signaling through its redox functional domain in pancreatic cancer cells. Int J Oncol. 2015;47: 610–620.

212. Cesaratto L, Codarin E, Vascotto C, Leonardi A, Kelley MR, Tiribelli C, *et al*. Specific inhibition of the redox activity of ape1/ref-1 by E3330 blocks TNF-α-induced activation of IL-8 production in liver cancer cell lines. PLoS One. 2013;8: e70909.

213. Jiang A, Gao H, Kelley MR, Qiao X. Inhibition of APE1/Ref-1 redox activity with APX3330 blocks retinal angiogenesis in vitro and in vivo. Vision Res. 2011;51: 93–100.

214. Li Y, Liu X, Zhou T, Kelley MR, Edwards P, Gao H, *et al*. Inhibition of APE1/Ref-1 redox activity rescues human retinal pigment epithelial cells from oxidative stress and reduces choroidal neovascularization. Redox Biol. 2014;2: 485–494.

215. Li Y, Liu X, Zhou T, Kelley MR, Edwards PA, Gao H, *et al*. Suppression of choroidal neovascularization through inhibition of APE1/Ref-1 redox activity. Invest Ophthalmol Vis Sci. 2014;55: 4461–4469.

216. Raffoul JJ, Banerjee S, Singh-Gupta V, Knoll ZE, Fite A, Zhang H, *et al*. Down-regulation of apurinic/apyrimidinic endonuclease 1/redox factor-1 expression by soy isoflavones enhances prostate cancer radiotherapy in vitro and in vivo. Cancer Res. 2007;67: 2141–2149.

217. Luo M, He H, Kelley MR, Georgiadis MM. Redox regulation of DNA repair: implications for human health and cancer therapeutic development. Antioxid Redox Signal. 2010;12: 1247–1269.

218. Bray F, Jemal A, Grey N, Ferlay J, Forman D. Global cancer transitions according to the Human Development Index (2008-2030): a population-based study. Lancet Oncol. 2012;13: 790–801.

219. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, *et al*. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J cancer. 2015;136: E359–E386.

220. Tell G, Damante G, Caldwell D, Kelley MR. The intracellular localization of APE1/Ref-1: more than a passive phenomenon? Antioxid Redox Signal. 2005;7: 367–384.

221. Scott TL, Wicker CA, Suganya R, Dhar B, Pittman T, Horbinski C, *et al*. Polyubiquitination of apurinic/apyrimidinic endonuclease 1 by Parkin. Mol Carcinog. 2016; doi:10.1002/mc.22495

222. Chattopadhyay R, Wiederhold L, Szczesny B, Boldogh I, Hazra TK, Izumi T, *et al*. Identification and characterization of mitochondrial abasic (AP)-endonuclease in mammalian cells. Nucleic Acids Res. 2006;34: 2067–2076.

223. Kakolyris S, Kaklamanis L, Engels K, Turley H, Hickson ID, Gatter KC, *et al*. Human Apurinic Endonuclease 1 expression in a colorectal adenoma-carcinoma sequence. Cancer Res. 1997;57: 1794–1797.

224. Di Maso V, Avellini C, Crocè LS, Rosso N, Quadrifoglio F, Cesaratto L, *et al*. Subcellular localization of APE1/Ref-1 in human hepatocellular carcinoma: possible prognostic significance. Mol Med. 2007;13: 89–96.

225. Moore DH, Michael H, Tritt R, Parsons SH, Kelley MR. Alterations in the expression of the DNA repair/redox enzyme APE/ref-1 in epithelial ovarian cancers. Clin Cancer Res. 2000;6: 602–609.

226. Al-Attar A, Gossage L, Fareed KR, Shehata M, Mohammed M, Zaitoun AM, *et al*. Human apurinic/apyrimidinic endonuclease (APE1) is a prognostic factor in ovarian, gastro-oesophageal and pancreatico-biliary cancers. Br J Cancer. 2010;102: 704–709.

227. Sheng Q, Zhang Y, Wang R, Zhang J, Chen B, Wang J, *et al*. Prognostic significance of APE1 cytoplasmic localization in human epithelial ovarian cancer. Med Oncol. 2012;29: 1265–1271.

228. Tanner B, Grimme S, Schiffer I, Heimerdinger C, Schmidt M, Dutkowski P, *et al*. Nuclear expression of apurinic/apyrimidinic endonuclease increases with progression of ovarian carcinomas. Gynecol Oncol. 2004;92: 568–577.

229. Zhang Y, Wang J, Xiang D, Wang D, Xin X. Alterations in the expression of the apurinic/apyrimidinic endonuclease-1/redox factor-1 (APE1/Ref-1) in human ovarian cancer and indentification of the therapeutic potential of APE1/Ref-1 inhibitor. Int J Oncol. 2009;35: 1069–1079.

230. Jiang Y, Zhou S, Sandusky GE, Kelley MR, Fishel ML. Reduced expression of DNA repair and redox signaling protein APE1/Ref-1 impairs human pancreatic cancer cell survival, proliferation, and cell cycle progression. Cancer Invest. 2010;28: 885–895.

231. Koukourakis MI, Giatromanolaki A, Kakolyris S, Sivridis E, Georgoulias V, Funtzilas G, *et al*. Nuclear expression of human apurinic/apyrimidinic endonuclease (HAP1/Ref-1) in head-and-neck cancer is associated with resistance to chemoradiotherapy and poor outcome. Int J Radiat Oncol. 2001;50: 27–36.

232. Puglisi F, Aprile G, Minisini AM, Barbone F, Cataldi P, Tell G, *et al*. Prognostic significance of Ape1/ref-1 subcellular localization in non-small cell lung carcinomas. Anticancer Res. 2001;21: 4041–4049.

233. Wang D, Xiang D-B, Yang X-Q, Chen L-S, Li M-X, Zhong Z-Y, *et al*. APE1 overexpression is associated with cisplatin resistance in non-small cell lung cancer and targeted inhibition of APE1 enhances the activity of cisplatin in A549 cells. Lung Cancer. 2009;66: 298–304.

234. Puglisi F, Barbone F, Tell G, Aprile G, Pertoldi B, Raiti C, *et al*. Prognostic role of Ape/Ref-1 subcellular expression in stage I-III breast carcinomas. Oncol Rep. 2002;9: 11–17.

235. Curtis CD, Thorngren DL, Nardulli AM. Immunohistochemical analysis of oxidative stress and DNA repair proteins in normal mammary and breast cancer tissues. BMC Cancer. 2010;10: 9.

236. Kakolyris S, Kaklamanis L, Engels K, Fox SB, Taylor M, Hickson ID, *et al*. Human AP endonuclease 1 (HAP1) protein expression in breast cancer correlates with lymph node status and angiogenesis. Br J Cancer. 1998;77: 1169–1173.

237. Kelley MR, Cheng L, Foster R, Tritt R, Jiang J, Broshears J, *et al*. Elevated and altered expression of the multifunctional DNA base excision repair and redox enzyme Ape1/ref-1 in prostate cancer. Clin Cancer Res. 2001;7: 824–830.

238. Wang D, Luo M, Kelley MR. Human apurinic endonuclease 1 (APE1) expression and prognostic significance in osteosarcoma: Enhanced sensitivity of osteosarcoma to DNA damaging agents using silencing RNA APE1 expression inhibition. Mol Cancer Ther. 2004;3: 679–686.

239. Schena M, Guarrera S, Buffoni L, Salvadori A, Voglino F, Allione A, *et al*. DNA repair gene expression level in peripheral blood and tumour tissue from non-small cell lung cancer and head and neck squamous cell cancer patients. DNA Repair (Amst). 2012;11: 374–380.

240. Sak SC, Harnden P, Johnston CF, Paul AB, Kiltie AE. APE1 and XRCC1 protein expression levels predict cancer-specific survival following radical radiotherapy in bladder cancer. Clin Cancer Res. 2005;11: 6205–6211.

241. Qing Y, Li Q, Ren T, Xia W, Peng Y, Liu G-L, *et al*. Upregulation of PD-L1 and APE1 is associated with tumorigenesis and poor prognosis of gastric cancer. Drug Des Devel Ther. 2015;9: 901–909.

242. Robertson KA, Bullock HA, Xu Y, Tritt R, Zimmerman E, Ulbright TM, *et al*. Altered expression of Ape1/ref-1 in germ cell tumors and overexpression in NT2 cells confers resistance to bleomycin and radiation. Cancer Res. 2001;61: 2220–2225.

243. Shin JH, Choi S, Lee YR, Park MS, Na YG, Irani K, *et al*. APE1/Ref-1 as a serological biomarker for the detection of bladder cancer. Cancer Res Treat. 2015;47: 823–833.

244. Silber JR, Bobola MS, Blank A, Schoeler KD, Haroldson PD, Huynh MB, *et al*. The apurinic/apyrimidinic endonuclease activity of Ape1/Ref-1 contributes to human glioma cell resistance to alkylating agents and is elevated by oxidative stress. Clin Cancer Res. 2002;8: 3008–3018.

245. Walker LJ, Craig RB, Harris AL, Hickson ID. A role for the human DNA repair enzyme HAP1 in cellular protection against DNA damaging agents and hypoxic stress. Nucleic Acids Res. 1994;22: 4884–4889.

246. Vasko MR, Guo C, Kelley MR. The multifunctional DNA repair/redox enzyme Ape1/Ref-1 promotes survival of neurons after oxidative stress. DNA Repair (Amst). 2005;4: 367–379.

247. Montaldi AP, Godoy PRDV, Sakamoto-Hojo ET. APE1/REF-1 down-regulation enhances the cytotoxic effects of temozolomide in a resistant glioblastoma cell line. Mutat Res Toxicol Environ Mutagen. 2015;793: 19–29.

248. Bobola MS, Finn LS, Ellenbogen RG, Geyer JR, Berger MS, Braga JM, *et al*. Apurinic/apyrimidinic endonuclease activity is associated with response to radiation and chemotherapy in medulloblastoma and primitive neuroectodermal tumors. Clin Cancer Res. 2005;11: 7405–7414.

249. Lau JP, Weatherdon KL, Skalski V, Hedley DW. Effects of gemcitabine on APE/ref-1 endonuclease activity in pancreatic cancer cells, and the therapeutic potential of antisense oligonucleotides. Br J Cancer. 2004;91: 1166–1173.

250. Naidu MD, Mason JM, Pica R V, Fung H, Peña LA. Radiation resistance in glioma cells determined by DNA damage repair activity of Ape1/Ref-1. J Radiat Res. 2010;51: 393–404.

251. Fung H, Demple B. Distinct roles of Ape1 protein in the repair of DNA damage induced by ionizing radiation or bleomycin. J Biol Chem. 2011;286: 4968–4977.

252. Chen S, Xiong G, Wu S, Mo J. Downregulation of apurinic/apyrimidinic endonuclease 1/redox factor-1 enhances the sensitivity of human pancreatic cancer cells to radiotherapy in vitro. Cancer Biother Radiopharm. 2013;28: 169–176.

253. McNeill DR, Wilson DM 3rd. A dominant-negative form of the major human abasic endonuclease enhances cellular sensitivity to laboratory and clinical DNA-damaging agents. Mol Cancer Res. 2007;5: 61–70.

254. McNeill DR, Lam W, DeWeese TL, Cheng Y-C, Wilson DM 3rd. Impairment of APE1 function enhances cellular sensitivity to clinically relevant alkylators and antimetabolites. Mol Cancer Res. 2009;7: 897–906.

255. Hung RJ, Hall J, Brennan P, Boffetta P. Genetic polymorphisms in the base excision repair pathway and cancer risk: a HuGE review. Am J Epidemiol. 2005;162: 925–942.

256. Hadi MZ, Coleman MA, Fidelis K, Mohrenweiser HW, Wilson DM 3rd. Functional characterization of Ape1 variants identified in the human population. Nucleic Acids Res. 2000;28: 3871–3879.

257. De Ruyck K, Szaumkessel M, De Rudder I, Dehoorne A, Vral A, Claes K, *et al*. Polymorphisms in base-excision repair and nucleotide-excision repair genes in relation to lung cancer risk. Mutat Res. 2007;631: 101–110.

258. Canbay E, Agachan B, Gulluoglu M, Isbir T, Balik E, Yamaner S, *et al*. Possible associations of APE1 polymorphism with susceptibility and HOGG1 polymorphism with prognosis in gastric cancer. Anticancer Res. 2010;30: 1359–1364.

259. DeSantis C, Bray F, Ferlay J. International variation in female breast cancer incidence and mortality rates. Cancer Epidemiol Biomarkers Prev. 2015;24: 1495–1506.

260. Scully OJ, Bay B-H, Yip G, Yu Y. Breast Cancer Metastasis. Cancer Genomics Proteomics. 2012;9: 311–320.

261. Cardoso F, Harbeck N, Fallowfield L, Kyriakides S, Senkus E, ESMO Guidelines Working Group. Locally recurrent or metastatic breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2012;23 Suppl 7: vii11–vii19.

262. Cardoso F, Costa A, Norton L, Senkus E, Aapro M, André F, *et al*. ESO-ESMO 2nd international consensus guidelines for advanced breast cancer (ABC2). Ann Oncol. 2014;25: 1871–1888.

263. Senkus E, Kyriakides S, Ohno S, Penault-Llorca F, Poortmans P, Rutgers E, *et al*. Primary breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2015;26 Suppl 5: v8–30.

264. Eccles SA, Aboagye EO, Ali S, Anderson AS, Armes J, Berditchevski F, *et al*. Critical research gaps and translational priorities for the successful prevention and treatment of breast cancer. Breast Cancer Res. 2013;15: R92.

265. Polyak K. Heterogeneity in breast cancer. J Clin Invest. 2011;121: 3786–3788.

266. Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. Nature. 2012;490: 61–70.

267. Coskun E, Jaruga P, Reddy PT, Dizdaroglu M. Extreme expression of DNA repair protein apurinic/apyrimidinic endonuclease 1 (APE1) in human breast cancer as measured by liquid chromatography and isotope dilution tandem mass spectrometry. Biochemistry. 2015;54: 5787–5790.

268. Poletto M, Di Loreto C, Marasco D, Poletto E, Puglisi F, Damante G, *et al*. Acetylation on critical lysine residues of Apurinic/apyrimidinic endonuclease 1 (APE1) in triple negative breast cancers. Biochem Biophys Res Commun. 2012;424: 34–39.

269. Zhang Y, Newcomb PA, Egan KM, Titus-Ernstoff L, Chanock S, Welch R, *et al*. Genetic polymorphisms in base-excision repair pathway genes and risk of breast cancer. Cancer Epidemiol Biomarkers Prev. 2006;15: 353–358.

270. Wu B, Liu HL, Zhang S, Dong XR, Wu G. Lack of an association between two BER gene polymorphisms and breast cancer risk: a meta-analysis. PLoS One. 2012;7: e50857.

271. Smith TR, Levine EA, Freimanis RI, Akman SA, Allen GO, Hoang KN, *et al*. Polygenic model of DNA repair genetic polymorphisms in human breast cancer risk. Carcinogenesis. 2008;29: 2132–2138.

272. Kang H, Dai Z, Ma X, Ma L, Jin Y, Liu X, *et al*. A genetic variant in the promoter of APE1 gene (-656 T>G) is associated with breast cancer risk and progression in a Chinese population. Gene. 2013;531: 97–100.

273. Chang-Claude J, Popanda O, Tan XL, Kropp S, Helmbold I, von Fournier D, *et al*. Association between polymorphisms in the DNA repair genes, XRCC1, APE1, and XPD and acute side effects of radiotherapy in breast cancer patients. Clin Cancer Res. 2005;11: 4802–4809.

CHAPTER 2

AIM

The aim of this work is the evaluation of a strategy for the improvement of standard breast cancer treatment by combining the use of APE1 pharmacological inhibitors with conventional anticancer agents in a highly aggressive human breast cancer cell line (MDA-MB-231 cells). As mentioned in Chapter 1, APE1 is a multifunctional protein with critical roles in the DNA repair and in the reduction/oxidation signalling by modulating the activation of multiple transcription factors involved in cancer progression. These features render APE1 as a potential target for anticancer drugs design and development. To achieve this general aim the following specific objectives are proposed:

a) Characterisation of the cytotoxicity, genotoxicity and migration/invasiveness profiles of standard therapeutic drugs for MBC;

b) Assessment of the combinations of commercially available inhibitors of the endonuclease and redox function of APE1 with the current chemotherapeutic drugs used in MBC in terms of cytotoxicity, genotoxicity and migration/invasion modulation;

c) Identification of novel small-molecule inhibitors of APE1 endonuclease activity by a computer-aided drug design campaign;

d) Evaluation of the effect of the selected compounds in APE1 endonuclease activity;

e) Assessment of the cytotoxicity of active compounds in off-target cells, namely in non-tumour breast cells.

In this thesis, we expect to be able to highlight the relevance of the APE1 inhibitors to sensitise breast cancer cells to anticancer drugs and identify novel chemical scaffolds which can be further optimised leading to clinically useful compounds.

Therefore, the Chapter 3 describes the effects of MX, the most studied and commercially available indirect inhibitor of the APE1 endonuclease activity, in combination with Dox, an anthracycline antibiotic, in human breast cancer MDA-MB-231 cells. MDA-MB-231 cell line was selected because it is a representative *in vitro* model of MBC. In this context, Dox was the anticancer drug used since it is a DNA-damaging agent which also undergoes redox-cycling producing ROS and it has been widely used in the treatment of MBC. The cytotoxicity and genotoxicity of the APE1 inhibitor/Dox combination was assessed with complementary endpoints to evaluate the therapeutic potential of this modality of combined chemotherapy.

Chapter 4 reports the identification with a structure-based virtual screening (SBVS) approach based on docking molecular studies of chemical entities from the compounds

library of National Cancer Institute/Developmental Therapeutics Program (NCI/DTP) with ability to inhibit the APE1 endonuclease activity. The cytotoxicity of the most promising inhibitors of APE1 DNA repair activity was also evaluated in the non-tumourigenic human breast epithelial MCF10A cell line.

The APE1 redox function is also a promising target to be used in combination with standard chemotherapeutic drugs. Chapter 5 describes the effects of E3330, the most studied redox inhibitor of APE1, combined with DTX in MDA-MB-231 cells. DTX is a taxane drug widely used in MBC refractory to adjuvant anthracycline-based therapeutic regimens. The viability, proliferation, cell cycle profile, the migratory properties and invasion ability of MDA-MB-231 cells treated with both compounds were assessed to evaluate the impact of this novel therapeutic strategy based on the targeting of APE1 redox function with E3330.

CHAPTER 3

DIFFERENTIAL EFFECTS OF METHOXYAMINE ON DOXORUBICIN CYTOTOXICITY AND GENOTOXICITY IN MDA-MB-231 HUMAN BREAST CANCER CELLS

This Chapter was adapted from:

Guerreiro PS, Fernandes AS, Costa JG, Castro M, Miranda JP, Oliveira NG. Differential effects of methoxyamine on doxorubicin cytotoxicity and genotoxicity in MDA-MB-231 human breast cancer cells. Mutat Res. 2013; 757:140–147.

3.1. ABSTRACT

Pharmacological inhibition of DNA repair is a promising approach to increase the effectiveness of anticancer drugs. The chemotherapeutic drug Dox may act, in part, by causing oxidative DNA damage. The BER pathway affects the repair of many DNA lesions induced by ROS. MX is an indirect inhibitor of apurinic/apyrimidinic endonuclease 1 (APE1), a multifunctional BER protein. We have evaluated the effects of MX on the cytotoxicity and genotoxicity of Dox in MDA-MB-231 metastatic breast cancer cells. MX has little effects on the viability and proliferation of Dox-treated cells. However, as assessed by the cytokinesis-block micronucleus (CBMN) assay, MX caused a significant 1.4-fold increase ($P < 0.05$) in the frequency of micronucleated binucleated cells induced by Dox, and also altered the distribution of the numbers of micronuclei. The fluorescence probe dihydroethidium (DHE) indicated little production of ROS by Dox. Overall, our results suggest differential outcomes for the inhibition of APE1 activity in breast cancer cells exposed to Dox, with a sensitising effect observed for genotoxicity but not for cytotoxicity.

Keywords

DNA repair, methoxyamine, doxorubicin, base excision repair, micronuclei, APE1

3.2. INTRODUCTION

The effectiveness of many anticancer drugs depends on their ability to damage the DNA, generating lesions that ultimately induce cell death. Cancer cells are often resistant to DNA-damaging agents, due to increased activity of DNA repair pathways (reviewed in [1,2]). Study of these repair systems has led to the development of pharmacological inhibitors of DNA repair, with a view to overcoming resistance to anticancer therapies and improving clinical outcomes.

BER pathway is the main system acting to remove and replace oxidised and alkylated bases. BER also repairs AP sites, spontaneously generated as a result of cellular metabolism or formed during the repair of lesions imposed by exogenous genotoxicants (e.g., TMZ, MMS, IR, and H_2O_2). BER also plays a critical role in the repair of SSBs generated by the attack of ROS on deoxyribose and the excision of uracil bases from DNA (reviewed in [3,4]).

BER proceeds via five coordinated steps: recognition and excision of damaged or inappropriate bases by a lesion-specific DNA glycosylase; incision of the abasic site by APE1 or by the AP lyase function of a bifunctional glycosylase; removal of the 5'- or 3'-terminal blocking groups by a lyase or phosphodiesterase; DNA synthesis (DNA polymerase), for filling the resulting gap; and finally, nick sealing, carried out by DNA ligase [3,4]. APE1 is a promising target for cancer therapy, since this multifunctional protein is of great importance for the repair of AP sites [5]. This ubiquitous enzyme is involved in both BER sub-pathways [5], although APE-independent DNA repair mechanisms have also been reported [6,7]. Additionally, APE1 plays an important role as a nuclear reduction/oxidation signalling protein, modulating the activation of multiple factors involved in cell survival, proliferation, and cancer promotion, such as NF-κB, AP-1, HIF-1α, and p53 [5]. Moreover, the expression and the subcellular distribution of APE1 are altered in several tumours. These alterations have been correlated with aggressiveness and poor prognosis in breast cancer [8] and other solid tumours (colorectal, hepatocellular, and epithelial ovarian carcinomas and non-small cell lung cancer) [9–12].

MX, a small-molecule inhibitor of BER, covalently binds to the aldehyde group of an AP site in the DNA and consequently prevents APE-catalysed hydrolytic cleavage of the phosphodiester bond. AP sites blocked by MX are stable adducts that preclude repair carried out by the downstream enzymes of BER [13–15]. Thus, these intermediates promote the accumulation of SSBs and DSBs that may cause cell death. MX enhances

the cytotoxic effects of chemotherapeutic drugs, such as TMZ [15–17], BCNU [18], pemetrexed [19], and IdUrd, and it potentiates IdUrd-mediated radiosensitisation [20,21]. Currently, MX is one of the most promising inhibitors of APE1; it is being evaluated in clinical trials in combination with the anticancer agents TMZ [22,23], pemetrexed [24], and fludarabine. Nevertheless, there is little information on the effect of MX on other DNA-damaging chemotherapeutic drugs, such as Dox.

Dox, an anthracycline antibiotic, is widely used for treatment of haematological malignancies and solid tumours such as breast cancer. The multiple mechanisms of Dox action are still incompletely elucidated. The antitumour activity of Dox seems to be mainly mediated through its DNA-damaging effects, which can be a consequence of poisoning topoisomerase IIα, DNA binding and alkylation, establishment of DNA interstrand crosslinks, and formation of base damage and strand breaks induced by the generation of ROS. Dox may also interfere with DNA unwinding, DNA strand separation, and helicase activity (reviewed in [25,26]).

ROS, including superoxide anion ($O_2^{\bullet-}$) and H_2O_2, may be generated as a result of reductive bioactivation of the Dox quinone moiety. Besides redox cycling, the enhancement of intracellular ROS levels that follows drug exposure can also be a response to perturbations of metabolism [25]. Since Dox presents multiple mechanisms of DNA damage, which may depend on drug concentration and cell type [25–27], one anticipates that multiple DNA repair pathways are activated by Dox. In fact, NER, HR, and NHEJ have been implicated in the repair of Dox-DNA adducts and DSBs [28–30]. Importantly, oxidative DNA lesions and SSBs induced by Dox may be repaired primarily by the BER pathway [31].

In this Chapter, we evaluated the effects of MX on the cytotoxicity and genotoxicity of Dox in MDA-MB-231 human breast cancer cells. Cell viability and proliferation were assessed using complementary endpoints, a tetrazolium reduction assay (MTT assay), the crystal violet staining (CV) assay, and the colony-formation assay. Since Dox-induced ROS may be involved in the formation of DNA lesions recognized by APE1 and repaired by BER, the fluorescence probe DHE was used to evaluate the production of ROS by Dox. Finally, the CBMN assay was performed to assess chromosomal damage induced by Dox in the presence of MX. Overall, the results show differential outcomes following APE1 and BER inhibition in malignant breast cancer cells exposed to Dox.

3.3. MATERIALS AND METHODS

3.3.1. Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin solution, thiazolyl blue tetrazolium bromide (MTT), CV, phosphate-buffered saline (PBS; 0.01 M, pH 7.4), trypsin, Dox, methoxyamine hydrochloride (MX, purity 98%), (2E)-2-[(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)methylene] undecanoic acid (E3330, purity ≥98%), 7-nitro-1*H*-indole-2-carboxylic acid (CRT0044876, purity ≥98%), *tert*-butylhydroperoxide (TBHP), cytochalasin B, glutaraldehyde, and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Doxorubicin was dissolved in Milli-Q H_2O and stored at -20 ºC. A stock solution of MX (2 M) in PBS was prepared; pH was adjusted with NaOH until neutral; aliquoted and stored at -20 ºC. E3330 (10 mM) was dissolved in DMSO, aliquoted, and stored at -20 ºC. H_2O_2, Giemsa dye, ethanol, methanol, and acetic acid were obtained from Merck (Darmstadt, Germany). DHE was acquired from Molecular Probes (Eugene, OR, USA). For this probe, a 10 mM stock solution was prepared in DMSO, aliquoted, and stored under N_2 at -20 ºC.

3.3.2. Cell culture

The human breast cancer cell line MDA-MB-231 was obtained from ATCC (HTB-26) and cultured in monolayer in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Cells were maintained at 37 ^{0}C, under a humidified atmosphere containing 5% CO_2 in air.

3.3.3. MTT reduction assay

Cell viability was evaluated using the MTT reduction assay. In brief, MDA-MB-231 cells were seeded at a density of approximately 2,500-3,000 cells per well, in 200 µL culture medium, in 96-well plates and incubated for 22 h at 37 °C under a 5% CO_2 atmosphere. Cells were incubated for 2 h with MX (10 mM and 20 mM), E3330 (30 µM) or with both APE1 inhibitors. After pre-incubation with MX or E3330, Dox or H_2O_2 (300 µM) were added and MDA-MB-231 cells were incubated for 24 h. Following drug treatments, cells were washed with culture medium and allowed to grow for a further 72 h in drug-free medium. An additional protocol was carried out, consisting of evaluation of cell viability after a 96 h period of continuous exposure to APE1 inhibitors and Dox or H_2O_2. The final concentration of DMSO in the culture medium did not exceed 0.3% (v/v) during drug treatments. The MTT assay was performed as previously described

[32,33]. Absorbance values presented by MDA-MB-231 cell cultures without addition of any compound (control cultures) corresponded to 100% cell viability. The number of independent experiments is indicated in the figure legends. Eight replicate cultures were used in each independent experiment.

3.3.4. Crystal violet (CV) staining assay

The CV staining assay was carried out as a confirmatory assay. 2,000-2,500 cells were plated in culture medium (200 µL per well) in 96-well plates and incubated for 24 h at 37 °C, 5% CO_2. Exposures were performed as for the MTT assay. The CV assay was then carried out according to a previously described protocol [32,33]. Absorbance values for untreated cells (control) correspond to 100% viability. The number of independent experiments is indicated in the figure legends. Eight replicate cultures were used in each independent experiment.

3.3.5. Colony formation assay

The colony formation assay was performed according to Franken *et al* [34] with minor modifications. Briefly, MDA-MB-231 cells were seeded into 6-well plates at cell density between 100 and 2,000 cells per well and maintained in a humidified incubator for 16 h, 37 °C, 5% CO_2. Cells were exposed to MX (10 mM) for 2 h before treatment with Dox (12.5 or 100 nM). After 24 h incubation with MX and Dox, the medium was removed and each well was rinsed with 1.0 mL PBS before addition of drug-free medium. Cultures were incubated 10-12 days to form colonies, with medium changed twice weekly. Cells were then fixed and stained with 6% (v/v) glutaraldehyde and 0.5% (w/v) crystal violet for 30 min. Colony fixation-staining solution was removed, colonies were thoroughly rinsed with tap water, and plates were dried at room temperature overnight. Colonies ≥ 50 cells were visually scored by two investigators. Results are presented as surviving fraction relative to untreated cells. All experiments were performed in duplicate for each drug treatment and three independent experiments were carried out.

3.3.6. DHE fluorimetric assay

Approximately 4×10^4 cells per well were cultured for 24 h in 96-well plates (black-wall/clear-bottom, Costar 3603). Thereafter, the culture medium was replaced and cells were exposed to Dox (0.1, 0.5, 1, or 2 µM) in the presence of DHE (10 µM) for 3 h. *Tert*-butylhydroperoxide (TBHP; 2 mM) was used as positive control [32,33]. After treatment, cells were carefully washed with PBS. PBS (200 µL) was added to each well and the fluorescence was determined at 37 °C in a multi-mode microplate reader, using $\lambda_{excitation}$ = 485 nm and $\lambda_{emission}$ = 590 nm [32,33,35]. Results were expressed as

percentages of untreated control cells, after subtracting background fluorescence. The number of independent experiments is indicated in the figure legend, each comprising four replicate cultures for each experimental point.

3.3.7. Cytokinesis-block micronucleus (CBMN) assay

3.3.7.1. Experimental protocol

Approximately 5,000 MDA-MB-231 cells were seeded in culture medium, 500 µL per well, in 8-well Lab-Tek™ II Chamber Slide™ System (Nunc) and incubated for 22 h at 37 °C. Afterwards, the cells were incubated with MX (20 mM) for 2 h and Dox (12.5 nM) was then added to the medium. Cells were grown for a further 24 h. After the treatment, cells were washed with culture medium, and cytochalasin B was added at a final concentration of 3.0 µg/mL to arrest cytokinesis [36]. Cells were allowed to grow for a further 28 h. MDA-MB-231 cells were then rinsed with PBS and the slides were fixed with ice-cold methanol for 20 min at -20 °C. After air drying, the Lab-Tek™ II Chamber Slide™ Systems were dismantled and slides were stained with Giemsa (4% v/v in 0.01 M phosphate buffer, pH 6.8) for 8 min. The slides were then coded for further microscopic analysis. Three independent experiments were performed for each drug treatment.

3.3.7.2. Micronucleus (MN) scoring

For the assessment of MN frequency, 1,000 binucleated (BN) cells with well-preserved cytoplasm were scored using 1250x magnification on a light microscope (Leitz), according to described criteria [36]. The frequency of micronucleated cells (% MNBN) present in 1,000 BN cells was used as the genotoxicity index. This index represents the frequency of DNA damaged cells (micronucleated) regardless of the number of micronuclei present in each damaged BN cell [37]. Moreover, the distribution of BN cells according to the number of MN and the total number of MN were also recorded [38].

3.3.7.3. Cell proliferation assessment

The decrease in cell proliferation for the experiments describe above (CBMN assay) was evaluated by two standard indices, the percentage of binucleated cells (% BN) and the nuclear division index (NDI) [36,37,39]. For these indices, 500 cells were classified according to the number of nuclei using a 1250x magnification in a light microscope (Leitz).

3.3.8. Statistical analysis

All values presented correspond to mean values and standard deviations (SD). The Kolmogorov-Smirnov test was used to assess the normality of continuous variables. For variables with normal distribution, homogeneity of variances was evaluated using the Levene test and the differences in mean values of the results observed in cultures with different treatments were evaluated by Student's t-test. For non-normal variables, the nonparametric Mann-Whitney test was used. All statistical analyses were performed using SPSS statistical package (version 21, SPSS Inc., Chicago, IL, USA) and the level of significance considered was $P < 0.05$.

3.4. RESULTS

3.4.1. Cytotoxicity profile of Dox in MDA-MB-231 cells

The effect of Dox on cell viability was first assessed by the MTT reduction assay (mitochondrial activity, Fig. 3.1). The CV staining (cell biomass) was used as a confirmatory assay (data not shown). In these experiments, MDA-MB-231 cells were treated with Dox for 24 h and then incubated in drug-free medium for 72 h. The dose-response profiles from both assays were roughly similar. Exposure to Dox concentrations up to 5 µM induced a concentration-dependent decrease in the cell viability, which was significant at Dox concentrations ≥ 50 nM ($P < 0.05$). The IC_{50} values obtained by non-linear regression were 328 nM and 259 nM for MTT and CV assays, respectively.

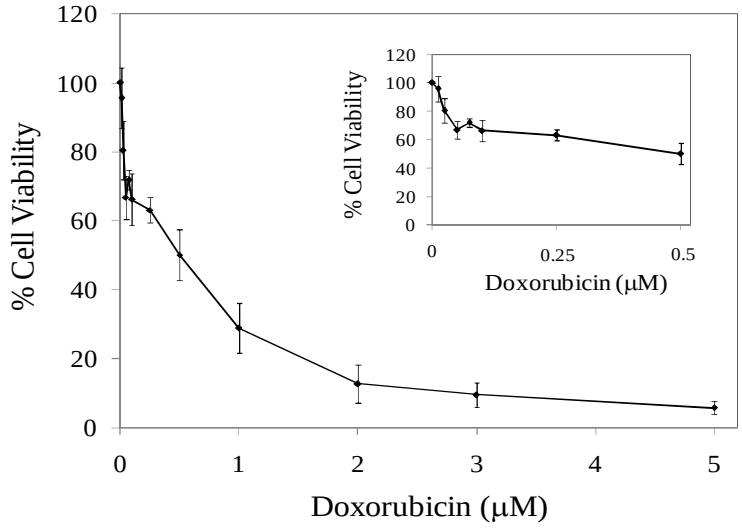


Figure 3.1 Cytotoxic effects of doxorubicin (Dox) in MDA-MB-231 cells. The decrease in the viability of Dox-treated cells (24 h) was evaluated 72 h after the exposure to Dox by MTT reduction (n= 2–14). Inset: Effect of low concentrations of Dox (up to 0.50 µM). Values represent mean ± SD and are expressed as percentages of the non-treated control cells.

3.4.2. Effect of MX on Dox cytotoxicity

The combination of MX (10 mM) with Dox (100 nM) showed a slight decrease in cell viability, as evaluated by the MTT assay when compared with cells treated with Dox (100 nM) only (Fig. 3.2A). A similar marginal effect of MX was observed in MDA-MB-231 cells exposed to the lower Dox concentration, 12.5 nM. For Dox 250 nM, cell viability was unchanged in presence of MX, 10 mM (Fig. 3.2A).

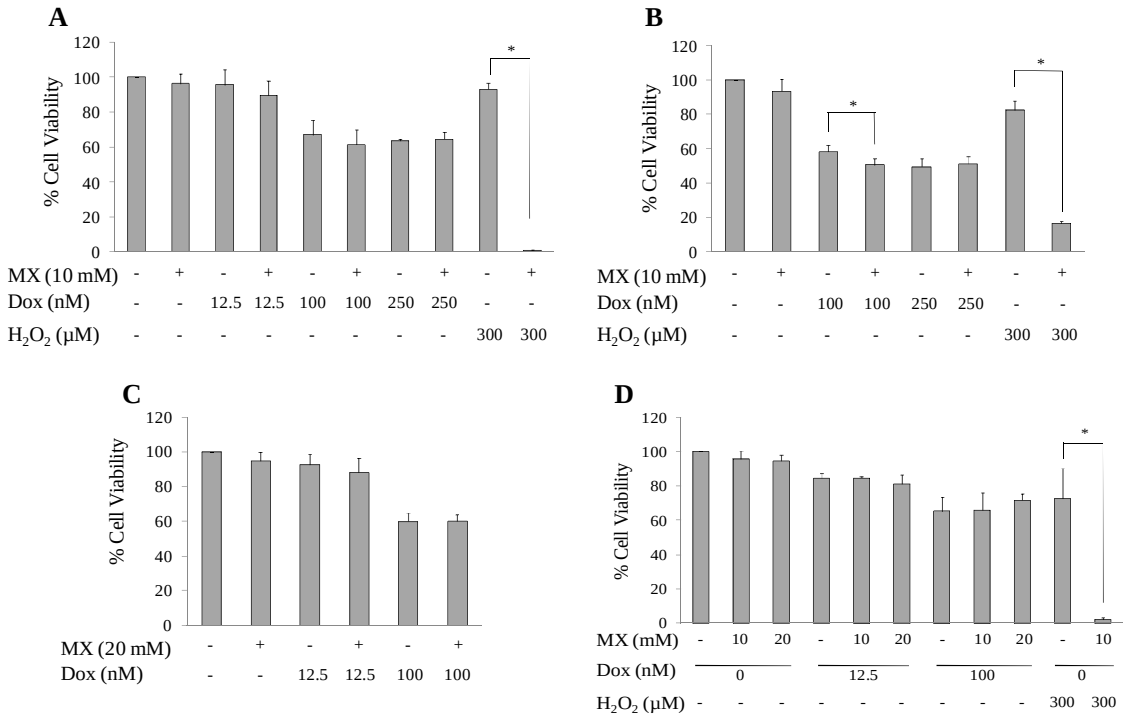


Figure 3.2 Doxorubicin-induced cytotoxicity in the presence of methoxyamine (MX) in MDA-MB-231 cells. Effect of MX (10 mM) in the viability of Dox-treated cells assessed by (A) MTT (n= 2–10) and (B) CV staining assays (n= 3–5). (C) Effect of MX (20 mM) in the viability of Dox-treated cells assessed by the MTT assay (n=4). In (A) to (C), MDA-MB-231 cells were pre-incubated for 2 h with MX and afterwards simultaneously exposed to Dox and MX for 24 h. Cells were then grown for a further period of 72 h in drug-free medium. (D) Effect of MX (10 and 20 mM) in the viability of Dox-treated cells assessed by the MTT assay. MX was pre-incubated for 2 h and cells were then simultaneously exposed to MX and Dox for a 96 h period (n= 3–4). H_2O_2 (300 μM) was used as a positive control (A, B and D). Values represent mean ± SD and are expressed as percentages relative to non-treated control cells. **P* < 0.05.

In the CV assay the small reduction in cell viability of cells treated with MX 10 mM and Dox 100 nM was confirmed (Fig. 3.2B). In this case, a decrease of approximately 8% in terms of cell viability was significant (*P* < 0.05). MDA-MB-231 cells were also exposed to H_2O_2 (300 μM), which is a well-known inducer of oxidative DNA lesions that can be repaired by BER [40–42]. As shown in Fig. 3.2A and 3.2B, cell viability was dramatically reduced (*P* < 0.05) upon the combination of MX with H_2O_2 as assessed by both MTT and CV assays. MX (10 mM) alone was not particularly toxic to MDA-MB-231 cells. In fact, this inhibitor decreased cell viability less than 5% for MTT (*P* < 0.05) and about 6% for the CV assay (N.S.). The effect of a higher concentration of MX (20 mM) towards the cytotoxicity of Dox (12.5 and 100 nM) using the MTT assay was further evaluated (Fig. 3.2C). The results revealed that the cell viability presented by Dox-treated cells was roughly in the same range in the presence or absence of MX.

The results presented in Fig. 3.2A–C were obtained using an experimental protocol that consisted in a pre-incubation with MX for 2 h, followed by a 24 h exposure to the combination of MX and Dox. Afterwards the cells were grown for a further 72 h period

in drug-free medium prior to the assessment of cell viability. In addition, a protocol with continuous exposure to both MX and Dox for 96 h was also included. These results showed that the cytotoxicity of Dox (12.5 nM and 100 nM) was not considerably altered by the presence of MX (10 mM and 20 mM) (Fig. 3.2D), as previously noted with the first treatment protocol. As in Fig. 3.2A and 3.B, MX 10 mM also showed a marked sensitising effect in the viability of MDA-MB-231 exposed to H_2O_2 300 µM ($P < 0.05$; Fig. 3.2D).

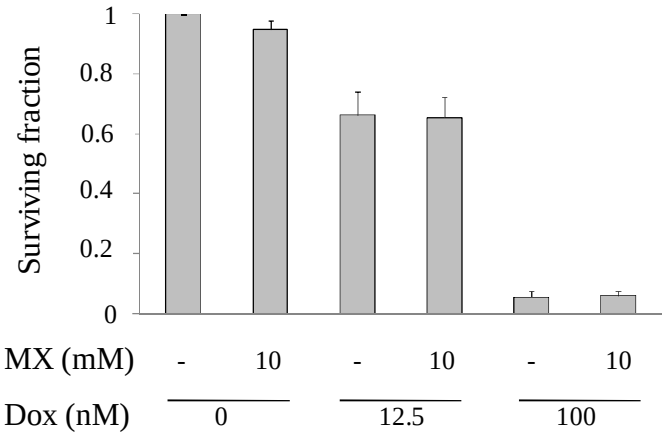


Figure 3.3 Colony formation assay of MDA-MB-231 cells treated with methoxyamine (MX) and doxorubicin (Dox). MDA-MB-231 cells were pre-incubated with MX (10 mM) for 2 h and then concomitantly exposed to Dox (12.5 and 100 nM) for 24 h. Cells were incubated in drug-free medium for 10-12 days to allow colony formation (n = 3). The surviving fraction after drug exposure was normalised to survival (plating efficiency) of non-treated control cells. Values represent mean ± SD.

Long-term effects of MX (10 mM) in the proliferation of MDA-MB-231 cells treated with Dox (12.5 and 100 nM) were assessed using the colony formation assay (Fig.3.3). Dox presented a more pronounced decrease in cell survival when compared with the effects in cell viability evaluated by the MTT reduction or CV assays (Fig. 3.1 and 3.2). MX *per se* only marginally reduced the colony forming ability when compared to non-treated controls (N.S.). Importantly, as shown in Fig. 3.3, MX did not affect the colony forming ability of Dox-treated MDA-MB-231 cells.

In order to evaluate the effect of simultaneous inhibition of DNA repair activity and the redox function of APE1 in the cytotoxicity of Dox, E3330, a well-known redox inhibitor of APE1 was used [43–47]. The results presented in Fig. 3.4 show that E3330 (30 µM) alone or in combination with MX 10 mM was not cytotoxic to MDA-MB-231 cells in both treatment protocols. Moreover, the simultaneous inhibition of DNA repair function and redox activity of APE1 also did not show a sensitising effect in the cytotoxicity of Dox (100 nM) as assessed by the MTT reduction assay (Fig. 3.4).

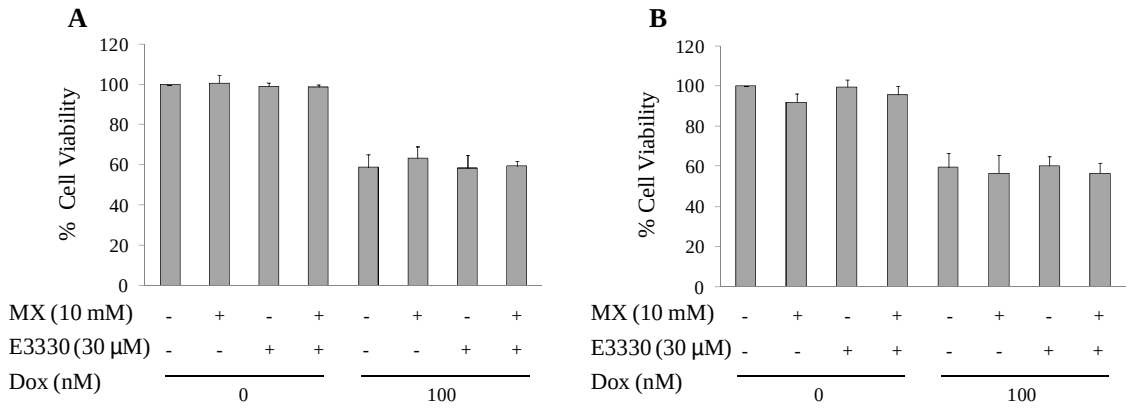


Figure 3.4 Effect of the simultaneous inhibition of the DNA repair activity and redox domain of APE1 in the viability of MDA-MB-231 cells treated with doxorubicin (Dox) as assessed by the MTT assay. Cells were pre-incubated with MX (10 mM) and E3330 (30 µM) for 2 h and then concomitantly exposed to Dox for (A) 24 h followed by a 72 hours period in drug-free medium (n= 3-4) or (B) a continuous 96 h period (n= 3). Values represent mean ± SD and are expressed as percentages relative to non-treated control cells.

3.4.3. Dox-induced ROS in MDA-MB-231 cells

To evaluate the ability of Dox to generate ROS, particularly $O_2^{\bullet-}$, the DHE assay was performed. Cells were exposed to Dox (0.1-2 µM) for 3 h. Under these conditions, Dox did not induce noticeable cell death. Dox, 2 µM, led to a small but significant increase (~10%; $P < 0.05$) in the intracellular level of superoxide radical anion, as evaluated by the fluorescence resulting from the oxidation of DHE probe (Fig. 3.5). Lower concentrations of Dox did not increase superoxide radical formation. TBHP, a model of acute oxidative stress, was used as a positive control and induced an increase in fluorescence intensity (1.6-fold; data not shown), similarly to results previously reported by our group with other cells [32,33].

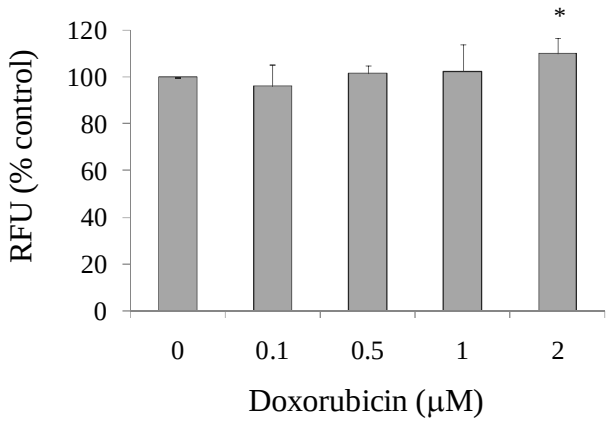


Figure 3.5 Effect of doxorubicin (Dox) in the intracellular levels of superoxide anion in MDA-MB-231 cells evaluated by the dihydroethidium (DHE) probe. Values (mean ± SD; n = 2-4) represent relative fluorescence units (RFU) and are expressed as percentages of non-treated control cells. *P< 0.05 when compared with control cells.

3.4.4. Assessment of the effect of MX in the genotoxicity of Dox

The results depicted in Fig. 3.6 indicate that the combination of MX (20 mM) with Dox (12.5 nM) did not affect cell proliferation at the concentrations selected, as observed by the absence of large alterations in the % BN (Fig. 3.6A) and NDI index (Fig. 3.6B). In view of this result, the effect of MX on the induction of micronuclei by Dox was evaluated at these concentrations.

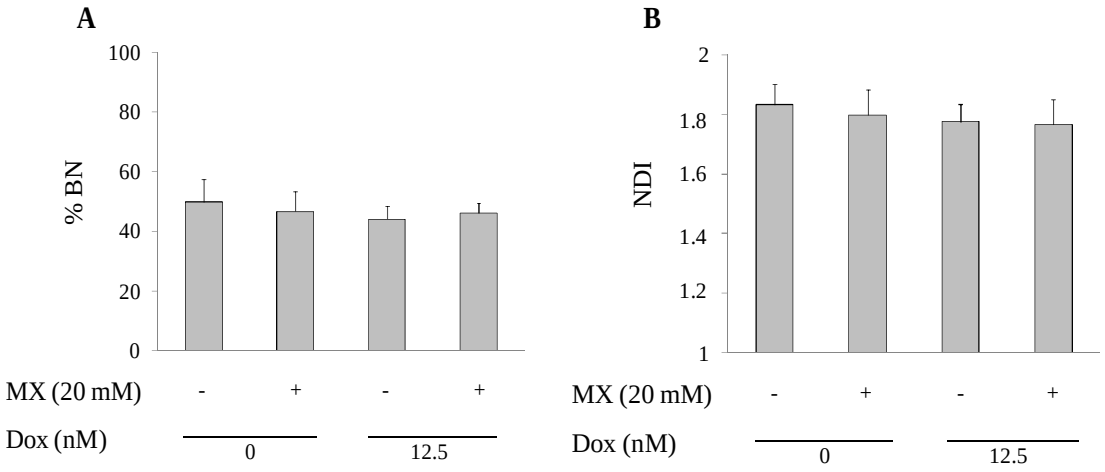


Figure 3.6 Effect of methoxyamine (MX) in the proliferative indices of Dox-treated cells associated with the cytokinesis-block micronucleus (CBMN) assay. MDA-MB-231 cells were pre-incubated for 2 h with MX (20 mM) and then simultaneously treated with Dox (12.5 nM) for 24 h. (A) the percentage of binucleated cells (% BN) and (B) nuclear divison index (NDI). Values represent mean ± SD (n= 3).

MDA-MB-231 cells presented a relatively high background frequency of MNBN cells (approximately 9%, Fig. 3.7) which may be a consequence of the intrinsic chromosomal instability of this cell line [48,49]. Moreover, MX seems to have no effect in the basal level of micronuclei (N.S.), suggesting an absence of genotoxicity under these conditions (Fig. 3.7). Dox was genotoxic, increasing the MNBN index from 8.90 to 17.27% ($P < 0.05$, Fig. 3.7A). Importantly, the combination of MX and Dox increased the frequency of micronucleated binucleated cells to 23.33%, which corresponds to a significant increase of ~ 1.4 fold when compared to MDA-MB-231 cells treated with Dox only ($P < 0.05$, Fig. 3.7A). A significant increase in the total number of micronuclei (1.5-fold) was also observed upon treatment with MX and Dox (data not shown). The sensitising effect observed was primarily due to an increase of BN cells with three or more MN ($P < 0.05$) as shown by the pattern of distribution of DNA damaged cells according to the number of MN (Fig. 3.7B).

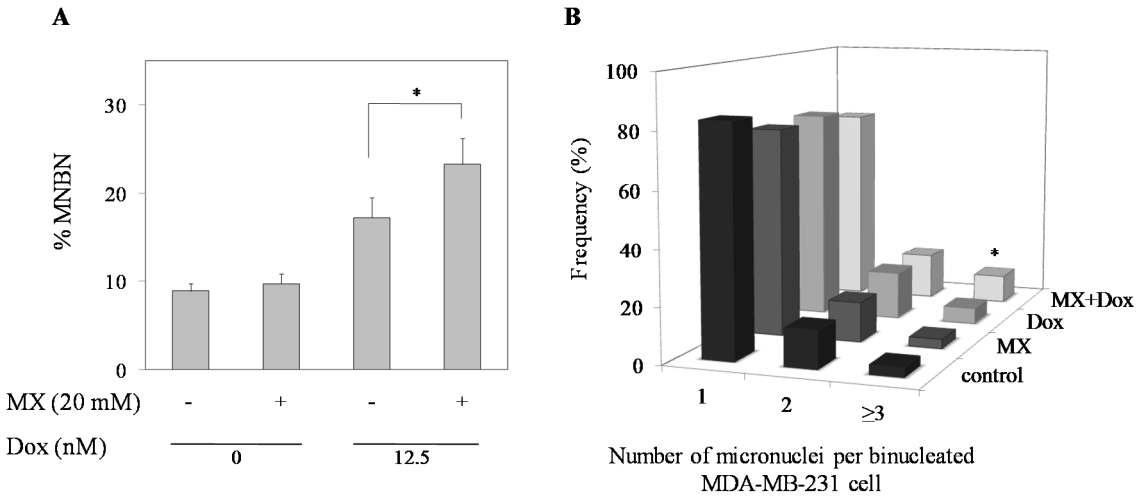


Figure 3.7 Effect of methoxyamine (MX) in Dox-induced micronuclei in MDA-MB-231 cells as evaluated by cytokinesis-block micronucleus (CBMN) assay. MDA-MB-231 cells were pre-incubated for 2 h with MX (20 mM) and then simultaneously treated with Dox (12.5 nM) for 24 h. (A) The frequency of micronucleated binucleated (% MNBN) cells and (B) distribution of the number of micronuclei per binucleated cell. Results are expressed as mean ± SD (n = 3). *P < 0.05 when compared with MDA-MB-231 cells treated only with Dox.

3.5. DISCUSSION

The effectiveness of a therapeutic strategy based on the pharmacological modulation of DNA repair proteins depends on the knowledge of the types of lesions induced by a given drug as well as on the DNA repair status of tumour cells. BER is primarily involved in the repair of damaged bases, abasic sites, and SSBs. These types of lesions have been identified or anticipated in the context of Dox exposure [50–52]. Among BER proteins, APE1 plays a pivotal role. We assessed the effect of MX, a standard inhibitor of APE1's DNA repair activity, on the cytotoxicity and genotoxicity of Dox in MDA-MB-231 breast cancer cells. This cell line was selected as a model of highly aggressive and metastatic breast cancer, a typical indication for Dox. APE1 was found to be increased after anthracycline-based therapy in post-surgical samples from breast tumours [53].

In order to evaluate the impact of MX in Dox-treated MDA-MB-231 cells, we first selected adequate Dox concentrations to be further used. For this purpose the cytotoxicity profiles of Dox in MDA-MB-231 cells were characterised using the MTT and CV assays. The protocol consisted of a 24 h incubation, approximately the duration of one cell cycle, followed by 72 h of growth in drug-free medium. This transient-exposure protocol has been adopted by other authors in cell-based assays [29,54] and resembles the clinical drug administration schedule. In the conditions described, MDA-MB-231 cells exhibited similar cytotoxicity profiles for both assays, with IC_{50} values in the same range as those described for MDA-MB-231 cells exposed to Dox for a 96 h period [55]. Moreover, Dox at 100 nM was selected as the primary concentration to be used in the viability assays in combination with MX. This concentration corresponded to a level of cell viability appropriate to study the potential modulator effect of MX, since much higher concentrations may lead to severe toxicity.

To test the effect of APE1 inhibition by MX, different experimental approaches were designed, including different Dox treatment schedules, MX concentrations and multiple endpoints, namely the MTT reduction and CV staining assays as well as the colony formation assay. This latter assay evaluates long-term effects on cell proliferation, and is considered by some to be the "gold standard" assay to study the effects of chemotherapeutic agents in tumour cells [56].

For both short-term MTT and CV assays, a minor effect in cell viability of MDA-MB-231 cells was detected following the exposure to MX, 10 mM, and Dox. Although MX at 10 mM or lower concentrations has been reported to significantly increase the formation of

abasic sites and to enhance the cytotoxic effect of distinct compounds [19,20,42,45], some authors suggested the use of higher MX concentrations in cell-based assays [15,17]. Thus, in an attempt to observe a more clear effect, we also studied MX at 20 mM. Also at this concentration, MX did not increase Dox cytotoxicity, demonstrating that the absence of a sensitising effect was not MX concentration-dependent.

Dox rapidly enters cells and, due to its lipophilic properties and affinity to DNA, it is trapped and accumulates in the intracellular compartment [26,57,58]. Since resistance to Dox may be related to a decrease in drug uptake or an increase in drug efflux, we also evaluated the effects of continuous exposure to MX and Dox. The results described above for the transient exposure protocol were recapitulated when MDA-MB-231 cells were exposed to both drugs continuously for 96 h. Moreover, and in respect to the colony formation assay, our results also showed an absence of sensitising effect by MX in Dox-treated cells. In these studies, Dox alone induced a decrease in colony formation more extensive than the effect observed in MTT and CV assays. These findings were anticipated, because Dox triggers senescence in different cell types [59,60], including MDA-MB-231 cells. This may explain the different sensitivity of MDA-MB-231 cells to Dox when different techniques are compared.

The work reported here focused on MX, since this compound is the reference inhibitor of APE1 activity, particularly relevant due to its use in clinical trials [22–24]. Nevertheless, we also tested the effect of a distinct BER inhibitor, the compound CRT0044876, which has been reported to directly inhibit APE1 [61]. Preliminary experiments using the MTT assay showed that APE1 inhibition by CRT0044876 did not modify the viability of Dox-treated MDA-MB-231 cells (data not shown). A recent study addressing the discovery of novel direct APE1 inhibitors towards different genotoxic agents also showed an absence of sensitising effects in melanoma and glioma cells treated with Dox [62].

Interestingly, the silencing of APE1 by short-hairpin RNA (shRNA) has been recently reported to increase the sensitivity of the human lung cancer H1299 cells towards Dox treatment, as well as to enhance the intracellular ROS generation and DNA damage [63]. In this context, and since E3330 is considered an effective inhibitor of the redox activity of APE1 [43–45], we also tested the effect of simultaneous inhibition of both DNA repair and redox functions of APE1. Although E3330 has been described to possess an endonuclease inhibitory activity, this was considered poor [46,47]. The concomitant treatment with MX and E3330, however, also did not improve the

sensitivity of MDA-MB-231 cells to Dox. These conflicting results may be a consequence of the distinct experimental approaches adopted.

Dox is an inducer of superoxide radical and other ROS [25,26,32]. While ROS have been considered the main responsible for Dox-induced cardiotoxicity [26,64], their contribution to the cytotoxic and genotoxic properties of Dox in cancer cells has been controversial, particularly at clinically relevant concentrations (up to 250 nM) [25,26]. Nevertheless, a few authors reported the presence of oxidised DNA bases after exposure to Dox concentrations achieved *in vivo* [51,52], which is important in the context of this work since this type of lesion can be repaired by the BER pathway. In an attempt to evaluate the intracellular production of ROS in breast cancer cells, the DHE assay was performed. The results showed a slight increase in the intracellular level of superoxide anion detected only at a high Dox concentration, these findings being consistent with other authors, who were able to detect ROS only at supraclinical Dox concentrations [25,65]. The apparent low contribution of ROS may partially explain the results from the MTT, CV and clonogenic assays. It is, however, important to note that Dox induces other types of lesions besides oxidised bases that could be repaired by the BER pathway, including SSBs [50].

A further aspect of this work was to evaluate whether an effect of MX could be observed at the chromosome damage level. In fact, since Dox is a DNA damaging drug, and a well-known clastogen, it seemed relevant to evaluate the effect of MX in terms of Dox-induced genotoxicity, specifically MN formation, using the CBMN assay [36,39]. The observed increase in the % MNBN along with the altered pattern of MN distribution pointed to an enhancement of damage severity by Dox upon BER inhibition. These findings suggest that MX promoted the accumulation of unrepaired lesions in Dox-treated MDA-MB-231 cells, increasing chromosomal damage. Importantly, this higher genotoxic burden was, however, not associated with a corresponding increment in cytotoxicity, which is ultimately the goal of chemotherapy. The reasons for this are unknown but these results suggest that the impairment of BER led to an increase in DNA lesions induced by Dox that can be somehow tolerated by MDA-MB-231 breast cancer cells. Although the correlation between survival and genotoxicity has been described, including for MN [66,67], there are also other reports addressing an apparent lack of correlation between these two events [68,69].

In summary, these results suggest a differential role of MX in terms of cytotoxicity and genotoxicity of Dox in human MDA-MB-231 breast cancer cells. Due to the absence of sensitising effects in cell viability and proliferation we can assume that a strategy based

on APE1 inhibition may not be relevant to enhance the efficacy of Dox in clinical terms. However, MX seems to be involved in the genotoxicity of Dox by a distinct mechanism. Therefore, the discrimination of the mechanisms underlying the effect of MX in Dox-induced cytotoxicity and genotoxicity should be further exploited in respect to cell death pathways and gene expression in different cell types.

3.6. REFERENCES

1. Helleday T, Petermann E, Lundin C, Hodgson B, Sharma RA. DNA repair pathways as targets for cancer therapy. Nat Rev Cancer. 2008;8: 193–204.
2. Rodrigues AS, Gomes BC, Martins C, Gromicho M, Oliveira NG, Guerreiro PS, *et al*. DNA repair and resistance to cancer therapy. In: Chen C, editor. New Research Directions in DNA Repair. InTech; 2013. pp. 489–528.
3. Izumi T, Wiederhold LR, Roy G, Roy R, Jaiswal A, Bhakat KK, *et al*. Mammalian DNA base excision repair proteins: their interactions and role in repair of oxidative DNA damage. Toxicology. 2003;193: 43–65.
4. Hegde ML, Hazra TK, Mitra S. Early steps in the DNA base excision/single-strand interruption repair pathway in mammalian cells. Cell Res. 2008;18: 27–47.
5. Bapat A, Fishel ML, Kelley MR. Going ape as an approach to cancer therapeutics. Antioxid Redox Signal. 2009;11: 651–668.
6. Das A, Wiederhold L, Leppard JB, Kedar P, Prasad R, Wang H, *et al*. NEIL2-initiated, APE-independent repair of oxidized bases in DNA: evidence for a repair complex in human cells. DNA Repair (Amst). 2006;5: 1439–1448.
7. Masani S, Han L, Yu K. Apurinic/apyrimidinic endonuclease 1 is the essential nuclease during immunoglobulin class switch recombination. Mol Cell Biol. 2013;33: 1468–1473.
8. Kakolyris S, Kaklamanis L, Engels K, Fox SB, Taylor M, Hickson ID, *et al*. Human AP endonuclease 1 (HAP1) protein expression in breast cancer correlates with lymph node status and angiogenesis. Br J Cancer. 1998;77: 1169–1173.
9. Kakolyris S, Kaklamanis L, Engels K, Turley H, Hickson ID, Gatter KC, *et al*. Human Apurinic Endonuclease 1 expression in a colorectal adenoma-carcinoma sequence. Cancer Res. 1997;57: 1794–1797.
10. Di Maso V, Avellini C, Crocè LS, Rosso N, Quadrifoglio F, Cesaratto L, *et al*. Subcellular localization of APE1/Ref-1 in human hepatocellular carcinoma: possible prognostic significance. Mol Med. 2007;13: 89–96.
11. Moore DH, Michael H, Tritt R, Parsons SH, Kelley MR. Alterations in the expression of the DNA repair/redox enzyme APE/ref-1 in epithelial ovarian cancers. Clin Cancer Res. 2000;6: 602–609.
12. Puglisi F, Aprile G, Minisini AM, Barbone F, Cataldi P, Tell G, *et al*. Prognostic significance of Ape1/ref-1 subcellular localization in non-small cell lung carcinomas. Anticancer Res. 2001;21: 4041–4049.
13. Liuzzi M, Talpaert-Borle M. A new approach to the study of the base-excision repair pathway using methoxyamine. J Biol Chem. 1985;260: 5252–5258.
14. Fortini P, Calcagnile A, Vrieling H, Zeeland AA van, Bignami M, Dogliotti E. Mutagenic processing of ethylation damage in mammalian cells: the use of methoxyamine to study apurinic/apyrimidinic site-induced mutagenesis. Cancer Res. 1993;53: 1149–1155.
15. Yan L, Bulgar A, Miao Y, Mahajan V, Donze JR, Gerson SL, *et al*. Combined treatment with temozolomide and methoxyamine: blocking apurininc/pyrimidinic site repair coupled with targeting topoisomerase IIalpha. Clin Cancer Res. 2007;13: 1532–1539.

16. Taverna P, Liu L, Hwang HS, Hanson AJ, Kinsella TJ, Gerson SL. Methoxyamine potentiates DNA single strand breaks and double strand breaks induced by temozolomide in colon cancer cells. Mutat Res. 2001;485: 269–281.

17. Fishel ML, He Y, Smith ML, Kelley MR. Manipulation of base excision repair to sensitize ovarian cancer cells to alkylating agent temozolomide. Clin Cancer Res. 2007;13: 260–267.

18. Liu L, Yan L, Donze JR, Gerson SL. Blockage of abasic site repair enhances antitumor efficacy of 1,3-bis-(2-chloroethyl)-1-nitrosourea in colon tumor xenografts. Mol Cancer Ther. 2003;2: 1061–1066.

19. Bulgar AD, Weeks LD, Miao Y, Yang S, Xu Y, Guo C, *et al*. Removal of uracil by uracil DNA glycosylase limits pemetrexed cytotoxicity: overriding the limit with methoxyamine to inhibit base excision repair. Cell Death Dis. 2012;3: e252.

20. Taverna P, Hwang HS, Schupp JE, Radivoyevitch T, Session NN, Reddy G, *et al*. Inhibition of base excision repair potentiates iododeoxyuridine-induced cytotoxicity and radiosensitization. Cancer Res. 2003;63: 838–846.

21. Yan T, Seo Y, Schupp JE, Zeng X, Desai AB, Kinsella TJ. Methoxyamine potentiates iododeoxyuridine-induced radiosensitization by altering cell cycle kinetics and enhancing senescence. Mol Cancer Ther. 2006;5: 893–902.

22. Savvides PS, Xu Y, Liu L, Rogers L, Bokar J, Silverman P, *et al*. Phase 1 clinical trial of the base-excision repair inhibitor methoxyamine-HCl (TRC102; MX) in combination with temozolomide (TMZ) in patients with solid tumors. Cancer Res. 2012;72 (Suppl 8):abstract no. 1743

23. Savvides PS, Xu Y, Liu L, Rogers L, Krishnamurthi S, Dowlati A, *et al*. Pharmacokinetic profile of the base-excision repair inhibitor methoxyamine-HCl (TRC102; MX) given as an one-hour intravenous infusion with temozolomide (TMZ) in the first in human phase 1 clinical trial. Cancer Res 2012;72(Suppl 8):abstract no. 753.

24. Gordon MS, Rosen LS, Mendelson D, Ramanathan RK, Goldman J, Liu L, *et al*. A phase 1 study of TRC102, an inhibitor of base excision repair, and pemetrexed in patients with advanced solid tumors. Invest New Drugs. 2013;31: 714–723.

25. Gewirtz DA. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. Biochem Pharmacol. 1999;57: 727–741.

26. Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol Rev. 2004;56: 185–229.

27. Müller I, Jenner A, Bruchelt G, Niethammer D, Halliwell B. Effect of concentration on the cytotoxic mechanism of doxorubicin--apoptosis and oxidative DNA damage. Biochem Biophys Res Commun. 1997;230: 254–257.

28. Spencer DMS, Bilardi RA, Koch TH, Post GC, Nafie JW, Kimura K-I, *et al*. DNA repair in response to anthracycline-DNA adducts: a role for both homologous recombination and nucleotide excision repair. Mutat Res. 2008;638: 110–121.

29. Saffi J, Agnoletto MH, Guecheva TN, Batista LFZ, Carvalho H, Henriques JAP, *et al*. Effect of the anti-neoplastic drug doxorubicin on XPD-mutated DNA repair-deficient human cells. DNA Repair (Amst). 2010;9: 40–47.

30. Davidson D, Grenier J, Martinez-Marignac V, Amrein L, Shawi M, Tokars M, *et al*. Effects of the novel DNA dependent protein kinase inhibitor, IC486241, on the DNA damage response to doxorubicin and cisplatin in breast cancer cells. Invest New Drugs. 2012;30: 1736–1742.

31. Jackson SP, Bartek J. The DNA-damage response in human biology and disease. Nature. 2009;461: 1071–1078.

32. Fernandes AS, Serejo J, Gaspar J, Cabral F, Bettencourt AF, Rueff J, *et al*. Oxidative injury in V79 Chinese hamster cells: protective role of the superoxide dismutase mimetic MnTM-4-PyP. Cell Biol Toxicol. 2010a;26: 91–101.

33. Fernandes AS, Gaspar J, Cabral MF, Rueff J, Castro M, Batinic-Haberle I, *et al*. Protective role of ortho-substituted Mn(III) N-alkylpyridylporphyrins against the oxidative injury induced by tert-butylhydroperoxide. Free Radic Res. 2010b;44: 430–440.

34. Franken NAP, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells in vitro. Nat Protoc. Nature Publishing Group; 2006;1: 2315–2319.

35. Gonçalves S, Fernandes AS, Oliveira NG, Marques J, Costa J, Cabral MF, *et al*. Cytotoxic effects of cadmium in mammary epithelial cells: protective role of the macrocycle [15]pyN5. Food Chem Toxicol. 2012;50: 2180–2187.

36. Fenech M. Cytokinesis-block micronucleus cytome assay. Nat Protoc. 2007;2: 1084–1104.

37. Ramos DL, Gaspar JF, Pingarilho M, Gil OM, Fernandes AS, Rueff J, *et al*. Genotoxic effects of doxorubicin in cultured human lymphocytes with different glutathione S-transferase genotypes. Mutat Res. 2011;724: 28–34.

38. Oliveira NG, Castro M, Rodrigues AS, Gonçalves IC, Cassapo R, Fernandes AP, *et al*. Evaluation of the genotoxic effects of the boron neutron capture reaction in human melanoma cells using the cytokinesis block micronucleus assay. Mutagenesis. 2001;16: 369–375.

39. Fenech M. The in vitro micronucleus technique. Mutat Res. 2000;455: 81–95.

40. Henle ES, Linn S. Formation, prevention, and repair of DNA damage by iron/hydrogen peroxide. J Biol Chem. 1997;272: 19095–19098.

41. Termini J. Hydroperoxide-induced DNA damage and mutations. Mutat Res. 2000;450: 107–124.

42. Jiang Y, Guo C, Fishel ML, Wang Z-Y, Vasko MR, Kelley MR. Role of APE1 in differentiated neuroblastoma SH-SY5Y cells in response to oxidative stress: use of APE1 small molecule inhibitors to delineate APE1 functions. DNA Repair (Amst). 2009;8: 1273–1282.

43. Shimizu N, Sugimoto K, Tang J, Nishi T, Sato I, Hiramoto M, *et al*. High-performance affinity beads for identifying drug receptors. Nat Biotechnol. 2000;18: 877–881.

44. Zou GM, Luo MH, Reed A, Kelley MR, Yoder MC. Ape1 regulates hematopoietic differentiation of embryonic stem cells through its redox functional domain. Blood. 2007;109: 1917–1922.

45. Zou GM, Maitra A. Small-molecule inhibitor of the AP endonuclease 1/REF-1 E3330 inhibits pancreatic cancer cell growth and migration. Mol Cancer Ther. 2008;7: 2012–2021.

46. Manvilla BA, Wauchope O, Seley-Radtke KL, Drohat AC. NMR studies reveal an unexpected binding site for a redox inhibitor of AP endonuclease 1. Biochemistry. 2011;50: 10540–10549.

47. Zhang J, Luo M, Marasco D, Logsdon D, LaFavers KA, Chen Q, *et al*. Inhibition of apurinic/apyrimidinic endonuclease I's redox activity revisited. Biochemistry. 2013;52: 2955–2966.

48. Maggiorella L, Deutsch E, Frascogna V, Chavaudra N, Jeanson L, Milliat F, *et al*. Enhancement of radiation response by roscovitine in human breast carcinoma in vitro and in vivo. Cancer Res. 2003;63: 2513–2517.

49. Yoon DS, Wersto RP, Zhou W, Chrest FJ, Garrett ES, Kwon TK, *et al*. Variable levels of chromosomal instability and mitotic spindle checkpoint defects in breast cancer. Am J Pathol. 2002;161: 391–397.

50. Capranico G, De Isabella P, Penco S, Tinelli S, Zunino F. Role of DNA breakage in cytotoxicity of doxorubicin, 9-deoxydoxorubicin, and 4-demethyl-6-deoxydoxorubicin in murine leukemia P388 cells. Cancer Res. 1989;49: 2022–2027.

51. Doroshow JH, Synold TW, Somlo G, Akman SA, Gajewski E. Oxidative DNA base modifications in peripheral blood mononuclear cells of patients treated with high-dose infusional doxorubicin. Blood. 2001;97: 2839–2845.

52. Gajewski E, Gaur S, Akman SA, Matsumoto L, van Balgooy JNA, Doroshow JH. Oxidative DNA base damage in MCF-10A breast epithelial cells at clinically achievable concentrations of doxorubicin. Biochem Pharmacol. 2007;73: 1947–1956.

53. Minisini AM, Di Loreto C, Mansutti M, Artico D, Pizzolitto S, Piga A, *et al*. Topoisomerase IIalpha and APE/ref-1 are associated with pathologic response to primary anthracycline-based chemotherapy for breast cancer. Cancer Lett. 2005;224: 133–139.

54. Azzariti A, Bocci G, Porcelli L, Fioravanti A, Sini P, Simone GM, *et al*. Aurora B kinase inhibitor AZD1152: determinants of action and ability to enhance chemotherapeutics effectiveness in pancreatic and colon cancer. Br J Cancer. 2011;104: 769–780.

55. Mansilla S, Priebe W, Portugal J. Mitotic catastrophe results in cell death by caspase-dependent and caspase-independent mechanisms. Cell Cycle. 2006;5: 53–60.

56. Brown JM, Wouters BG. Apoptosis, p53, and tumor cell sensitivity to anticancer agents. Cancer Res. 1999;59: 1391–1399.

57. van Wijngaarden J, van Beek E, van Rossum G, van der Bent C, Hoekman K, van der Pluijm G, *et al*. Celecoxib enhances doxorubicin-induced cytotoxicity in MDA-MB-231 cells by NF-kappaB-mediated increase of intracellular doxorubicin accumulation. Eur J Cancer. 2007;43: 433–442.

58. Tacar O, Sriamornsak P, Dass CR. Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems. J Pharm Pharmacol. 2013;65: 157–170.

59. Xu D, Shiu RP, Newsham IF, Gewirtz DA. Apoptosis, autophagy, accelerated senescence and reactive oxygen in the response of human breast tumor cells to adriamycin. BiochemPharmacol. 2009;77: 1139–1150.

60. Sliwinska MA, Mosieniak G, Wolanin K, Babik A, Piwocka K, Magalska A, *et al*. Induction of senescence with doxorubicin leads to increased genomic instability of HCT116 cells. Mech Ageing Dev. 2009;130: 24–32.

61. Madhusudan S, Smart F, Shrimpton P, Parsons JL, Gardiner L, Houlbrook S, *et al*. Isolation of a small molecule inhibitor of DNA base excision repair. Nucleic Acids Res. 2005;33: 4711–4724.

62. Mohammed MZ, Vyjayanti VN, Laughton CA, Dekker L V, Fischer PM, Wilson DM 3rd, *et al*. Development and evaluation of human AP endonuclease inhibitors in melanoma and glioma cell lines. Br J Cancer. 2011;104: 653–663.

63. Koedrith P, Seo YR. Sensitizing effect of silencing Ape1/Ref-1 on doxorubicin-induced apoptosis in human carcinoma cells. Mol Cell Toxicol. 2012;7: 389–397.

64. Zhou S, Palmeira CM, Wallace KB. Doxorubicin-induced persistent oxidative stress to cardiac myocytes. Toxicol Lett. 2001;121: 151–157.

65. Bernuzzi F, Recalcati S, Alberghini A, Cairo G. Reactive oxygen species-independent apoptosis in doxorubicin-treated H9c2 cardiomyocytes: role for heme oxygenase-1 down-modulation. Chem Biol Interact. 2009;177: 12–20.

66. Wandl EO, Ono K, Kain R, Herbsthofer T, Hienert G, Höbarth K. Linear correlation between surviving fraction and the micronucleus frequency. Int J Radiat Biol. 1989;56: 771–775.

67. Jagetia GC, Nayak V. Micronuclei-induction and its correlation to cell survival in HeLa cells treated with different doses of adriamycin. Cancer Lett. 1996;110: 123–128.

68. Champion AR, Hanson JA, Court JB, Venables SE. The micronucleus assay: an evaluation of its use in determining radiosensitivity in vitro. Mutagenesis. 1995;10: 203–208.

69. Villa R, Zaffaroni N, Gornati D, Costa A, Silvestrini R. Lack of a correlation between micronucleus formation and radiosensitivity in established and primary cultures of human tumours. Br J Cancer. 1994;70: 1112–1117.

CHAPTER 4

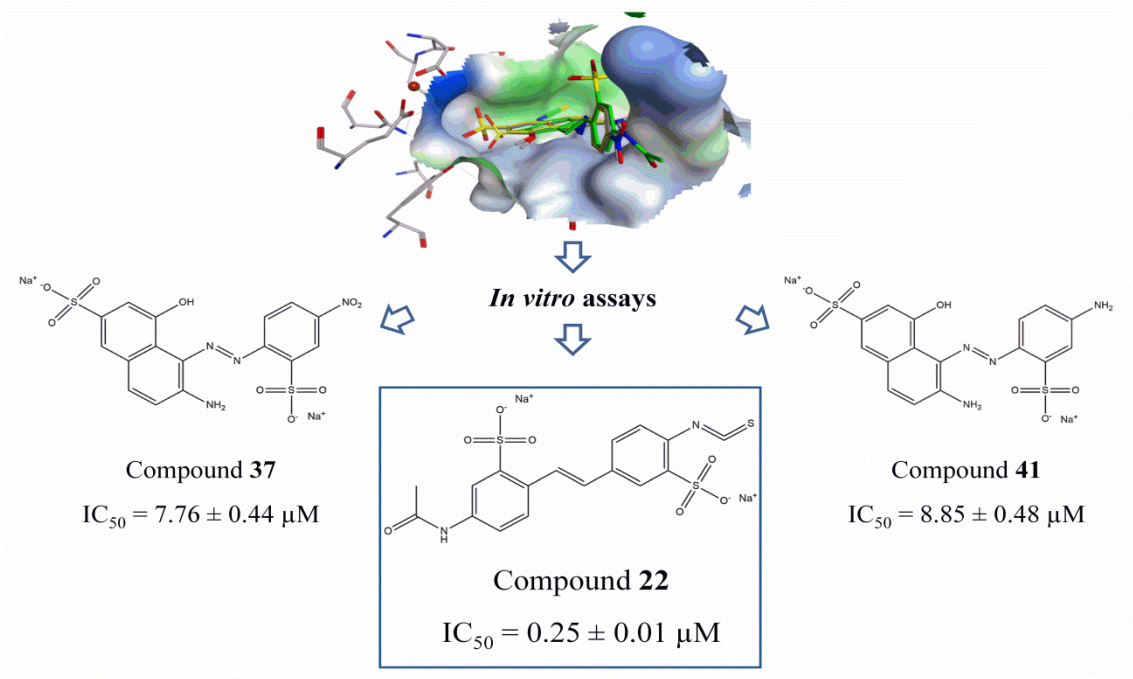
STRUCTURE-BASED VIRTUAL SCREENING TOWARDS THE DISCOVERY OF NOVEL INHIBITORS OF THE DNA REPAIR ACTIVITY OF THE HUMAN APURINIC/APYRIMIDINIC ENDONUCLEASE 1

This Chapter was adapted from:

Guerreiro PS, Estácio SG, Antunes F, Fernandes AS, Pinheiro PF, Costa JG, Castro M, Miranda JP, Guedes RC, Oliveira NG. Structure-based virtual screening toward the discovery of novel inhibitors of the DNA repair activity of the human apurinic/apyrimidinic endonuclease 1. Chem Biol Drug Des. 2016; 88:915-925.

4.1. ABSTRACT

The DNA repair activity of APE1 has been recognized as a promising target for the development of small-molecule inhibitors to be used in combination with anticancer agents. In an attempt to identify novel inhibitors of APE1 we present a SBVS study based on molecular docking analysis of the compounds of NCI/DTP database using the GOLD 5.1.0 (Genetic Optimization for Ligand Docking) suite of programs. Compounds selected in this screening were tested with a fluorescence-based APE1 endonuclease activity assay. Two compounds (37 and 41) were able to inhibit the multifunctional enzyme APE1 in the micromolar range while compound 22 showed inhibitory effects at nanomolar concentrations. These results were confirmed by a plasmid DNA nicking assay. In addition, the potential APE1 inhibitors did not affect the cell viability of non-tumour MCF10A cells. Overall, compounds 22, 37 and 41 appear to be important scaffolds for the design of novel APE1 inhibitors and this study highlights the relevance of *in silico* based approaches as valuable tools in drug discovery.



Keywords

Cancer, DNA damage, BER pathway, APE1 inhibitors, molecular docking, virtual screening, drug discovery.

4.2. INTRODUCTION

Multiple DNA repair pathways are involved in the repair of DNA lesions induced by standard chemotherapeutic drugs and radiotherapy. Therefore, the development of DNA repair inhibitors constitutes a promising approach for cancer therapy [1,2]. By inhibiting specific DNA repair enzymes, the therapeutic effect of a given agent in cancer cells could be improved, and drug resistance may even be overcome. The importance of DNA repair inhibitors has been extensively reviewed in the last decade [1,2]. Several cell-based experiments conducted with numerous small-molecule DNA repair inhibitors revealed important sensitising effects towards cytotoxic drugs from different classes [1–7] and IR [8–12]. Moreover, a few DNA repair inhibitors have been recently evaluated in clinical trials [13,14], which clearly emphasize the importance of this therapeutic strategy.

As mentioned in Chapters 1 and 3, BER is a key pathway responsible for the removal and replacement of oxidised and alkylated bases. This system is also involved in the repair of AP sites, SSBs and in the excision of uracil from DNA (reviewed in [15,16]). APE1 is a multifunctional enzyme that plays a fundamental role in BER, being responsible for the incision of the DNA phosphodiester backbone 5' to the AP site with the generation of terminal groups recognized and repaired by the downstream proteins of BER [15,17]. APE1 has other minor DNA repair functions which include 3'-phosphodiesterase, weak 3'-phosphatase, 3'-5' exonuclease and RNase H activity [15,17]. APE1 also acts as a nuclear reduction/oxidation signaling protein, modulating the activation of several transcription factors (*e.g.* NF-κB, AP-1, HIF-1α, p53 and others) implicated in cancer cell survival, proliferation, migration/invasion, angiogenesis and metastases formation (reviewed in [16,17]). In addition, alterations in the subcellular distribution and/or protein levels of APE1 have been associated to more aggressive tumour phenotypes with a poor prognosis and resistance to chemo/radiotherapy. These alterations have been found in a variety of tumours such as gliomas [18], hepatocellular [19], colorectal [20], prostate [21], breast [22], osteosarcoma [23] and non-small cell lung cancer [24]. In view of this, APE1 is considered an attractive key target for the discovery of novel DNA repair inhibitors.

Several APE1 inhibitors have been developed in the last years. MX, an indirect inhibitor [16,25,26] and CRT0044876, a direct inhibitor of the DNA repair activity of APE1, are the most usually studied compounds [27–29]. MX increased the cytotoxicity of chemotherapeutic drugs such as TMZ [7,26], BCNU [30], pemetrexed [6] and IdUrd [12] in preclinical models and it has been evaluated in clinical trials in combination with

alkylating or antimetabolite drugs in different tumour types [13,14]. Despite the promising effects reported, MX acts indirectly by reacting and binding to the AP sites in the DNA strand [25,26]. Direct enzyme inhibitors are usually preferable because they are more specific with a subsequent decrease of potential side effects. CRT0044876 was the first direct inhibitor of APE1 endonuclease activity identified by an *in vitro* HTS assay of a chemical library of 5000 “drug-like” compounds performed by Madhusudan *et al.* [27]. However, the role of CRT0044876 as an APE1 inhibitor is not clear and a few authors were not able to reproduce the *in vitro* results initially reported for this compound [29,31]. Lucanthone has also been regarded as a nonspecific direct inhibitor of APE1 since it also inhibits topoisomerase II and intercalates into the DNA [32,33]. Therefore, several authors proposed other classes of APE1 inhibitors using different approaches, which include the HTS of commercially available chemical libraries [29,34–36], the rationale design with structure-activity relationship studies [37] and also ligand-based pharmacophore models [28,38–41], and SBVS and molecular docking studies [28,42]. Nevertheless, there is still a need for additional studies to identify novel pharmacologically active APE1 inhibitors with clinical application as combination therapy in cancer treatment.

When the crystallographic structure of the target is available, SBVS is nowadays one of the most valuable tools for the identification of hit-compounds during the early stages of a drug discovery pipeline. This cost- and time-effective approach includes structure-based and ligand-based techniques. While the first one uses the information of the known or inferred 3D structure of the target and exploits the molecular recognition between a ligand and the residues of the target protein to identify chemical entities that bind strongly to the active sites, the second entails information of known ligands [43–45]. The aim of virtual screening is to speed up the initial stages of drug discovery, potentiating the rapid identification of hit molecules that can subsequently be biologically evaluated. Despite a relatively high number of false positives associated with virtual screening [46], the procedures are becoming progressively more accurate and the reliability of the results obtained is also increasing [47].

In this context, in this Chapter we report a successful computer aided drug design campaign combining a SBVS approach and molecular docking studies to the discovery of novel potent small-molecule inhibitors that target APE1 endonuclease activity (Fig. 4.1). NCI/DTP database containing 265 242 compounds (release 4, May 2012) was sequentially screened using GOLD 5.1.0 (Genetic Optimization for Ligand Docking) software [48,49]. The most promising compounds were tested with a fluorescence-based APE1 endonuclease activity assay and cytotoxicity was evaluated in non-tumour

MCF10A cells. Using our methodology we were able to identify novel small-molecule inhibitors with IC_{50} values in nanomolar (one compound) and low micromolar range (two compounds), that provide relevant chemical motifs for the rationale development of effective APE1 inhibitors.

4.3. MATERIALS AND METHODS

4.3.1. Chemicals

Human recombinant APE1 was obtained from New England Biolabs (Ipswich, MA, USA). Potential APE1 inhibitors and the arylstibonic acid NSC 13755 were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program of National Cancer Institute (Bethesda, MD, USA). Stock solutions of the candidate inhibitors were prepared in DMSO at a final concentration of 10 mM, aliquoted and stored at -20 °C. The structure of active compounds was confirmed by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) (Annex, Materials and methods S1). Compound purity was determined by high-performance liquid chromatography with a diode array detector (HPLC-DAD) and found to be higher than 90% in all cases. The 17-mer oligonucleotides 5'-TAMRA-TC ACC *TC GTA CGA CTC-3' and 3'-BHQ-2-AG TGG GAG CAT GCT GAG-5' (in which TAMRA is the fluorophore carboxytetramethylrhodamine, BHQ-2 is black hole quencher-2 and * is tetrahydrofuran, a stable AP site analogue) [35] were custom-made by NZYTech (Lisbon, Portugal). Both oligonucleotides were dissolved in TE buffer (1 mM EDTA, 10 mM Tris-HCl pH 8.0) at a concentration of 100 µM, aliquoted and stored at -20 °C.

Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM/F-12), horse serum (HS), penicillin-streptomycin solution and trypsin were purchased from Gibco® (Life Technologies, Madrid, Spain). Phosphate buffered saline (PBS) 0.01 M, pH 7.4, recombinant human insulin, hydrocortisone, cholera toxin, human epidermal growth factor, dimethylsulfoxide (DMSO) were acquired from Sigma-Aldrich (St. Louis, MO, USA).

4.3.2. Virtual screening library

A library of 265 242 compounds retrieved from the NCI repository was screened in this work. This collection of compound structures was built and is maintained by the Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis of the National Cancer Institute, National Institute of Health (Bethesda, MD, USA).

4.3.3. Structure-based virtual screening (SBVS) and molecular docking studies

A SBVS protocol based on molecular docking studies of the NCI compounds database using the GOLD 5.1.0 suite of programs [48,49] was performed to identify potential

inhibitors of APE1. All the compounds included in this library were previously protonated (at pH = 7 and 300 K) and partial charges were assigned using MMFF94x force field as implemented in the Molecular Operating Environment (MOE) 2011.10 software package [50]. Compounds for which initially attributed protonation states were found to be incorrect were further energy minimized ahead of the protonation procedure. GOLD package has implemented a genetic algorithm (GA) that performs searches for the best ligand interaction/binding poses which are then ranked in agreement with a defined scoring function. After testing several scoring functions, the GoldScore scoring function [48,49,51] was selected.

In order to prepare the enzyme structure for the SBVS calculations, all atoms other than the receptor were deleted from PDB 1BIX except for the metal (Sm^{3+}) in the active site which coordinates were conserved. The ion (Sm^{3+}) was later replaced by the preferred metal cofactor of the human APE1 enzyme, Mg^{2+}, by using the Mutate Residue functionality of MOE 2011.10 [50]. The AMBER99 force field [52] was then used to assign atom types and charges to each atom in the receptor. Hydrogen atoms were added and the protonation states assigned using the Protonate-3D tool within MOE 2011.10 software package [50]. The histidine residue His309 at the binding site was protonated in agreement with the observations in the NMR study performed by Lowry *et al.*[53]. The enzyme structure was then energy minimized in MOE with the AMBER99 force field for a final backbone root mean square deviation (RMSD) from the original X-ray structure of 0.997 Å. In order to validate this refined APE1 structure for the SBVS calculations, two well-known inhibitors of APE1 – lucanthone and hycanthone [32] – were docked into the targeted enzyme active site. The binding site was defined to be circumscribed by the amino acids Asp70, Glu96, Arg177, His309, Asp210, Asn212, Trp280, Phe266, and Leu282 with a 10 Å radius. Each of the latter compounds was constructed and had its energy minimized with the GizMoe minimizer in MOE (MMFF94x force field). The terminal amine was protonated in both cases according to what is predicted in the literature [32]. Docking calculations were performed with standard settings and 500 GA operations.

In the present study, SBVS of the NCI database was performed with speed-up settings, 50 GA operations/runs and 30% search efficiency. The top 1000 highest ranked compounds in SBVS were selected for posterior molecular docking (final refinement) studies (Figure 4.1). Refining molecular docking studies were performed using the GoldScore fitness scoring function with 500 GA runs and a search efficiency of 100% to select the representative pose of each compound. The final GoldScore scores were ranked and 200 compounds were considered for subsequent *in vitro* evaluation but all

the selected compounds were subjected to visual inspection using specific criteria: similar docking poses to hycanthone and lucanthone described by Naidu *et al.* [32]. After visual inspection, potential inhibitors with appropriate pocket fitting were selected to be acquired and evaluated for activity against human APE1.

4.3.4. Fluorescence-based APE1 endonuclease activity assay

The fluorescence-based APE1 endonuclease activity assay was performed as described previously by Simeonov *et al.* [35] with modifications. In this methodology, the fluorescence of TAMRA in the double-stranded DNA is quenched due to the proximity of the BHQ-2 quencher. When APE1 cleaves the double-stranded DNA at the position 5' of the AP site, a small single-stranded DNA fragment containing the fluorophore TAMRA is released, resulting in an increase in fluorescence. The presence of APE1 inhibitors should prevent this reaction.

In this assay, the complementary single-stranded oligonucleotides at 100 μM were mixed at a 1:1 ratio and annealed in assay buffer (50 mM Tris pH 7.5, 25 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 0.01% Tween-20) at 95 °C for 5 min to generate the double-stranded substrate of APE1 [35]. After the annealing, the reaction mixture was allowed to cool to room temperature. The assay was carried out in 96-well black plates with flat bottom (Costar®, Corning Inc., NY, USA) at a final reaction volume of 100 μL. The reaction mixtures containing 2.5 U of human recombinant APE1 (6 μL) were incubated in the assay buffer abovementioned, in the absence or presence of the putative APE1 inhibitors (5 μL), at room temperature for 15 min. The reaction was initiated by the addition of double-stranded substrate (6 μL) to obtain the final concentration of 250 nM. DMSO was maintained at a concentration of 0.5% (v/v) in all assays (negative control). Fluorescence readings were acquired at 1 min intervals over an incubation period of 30 min at 37 °C using a SpectraMax Gemini™ EM microplate plate reader (Molecular Devices, Berkshire, UK) in the kinetic mode at excitation wavelength 550 nm, emission wavelength 584 nm and cut-off at 570 nm. For each reaction, the rate values obtained from the tangent to the linear range of the progress curves were used to calculate the percentage of APE1 endonuclease activity. The reaction of APE1 in the presence of DMSO 0.5% (v/v) was considered to present 100% of activity (negative control). APE1 activity in the presence of compounds was calculated relatively to the negative control. The arylstibonic acid NSC 13755 was used as a positive control for APE1 endonuclease activity inhibition. The potential APE1 inhibitors identified from the SBVS/docking studies were first screened at a final concentration of 50 μM in duplicate and two independent experiments were performed for each compound. Those

compounds showing more than 85% inhibition of APE1 endonuclease activity were selected for IC_{50} determination. GraphPad Prism® 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to estimate the IC_{50} values, by fitting the data to a four-parameter logistic equation. Each value is presented as the mean and the SD of three to seven experiments performed in triplicate.

4.3.5. Fluorescence quenching assay

The interference of the identified compounds with the fluorescence signal of the fluorophore TAMRA was evaluated to discard false positive hits. The 17-mer oligonucleotide 5' TAMRA-TC ACC *TC GTA CGA CTC-3', at a final concentration of 250 nM, was incubated with potential APE1 inhibitors in assay buffer (50 mM Tris pH 7.5, 25 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 0.01% Tween-20). Fluorescence readings were obtained at 1 min intervals over 30 min of incubation at 37 °C as abovementioned.

4.3.6. Plasmid DNA nicking assay

A plasmid DNA nicking assay was performed to confirm the ability of identified compounds to inhibit APE1's endonuclease activity [32,54]. This assay evaluates the conversion of supercoiled plasmid DNA to a relaxed form by APE1 incision at an AP site [32,54]. Plasmid pcDNA3.1 (+) was amplified in *Escherichia coli* strain DH5α and extracted using NZYMidiprep kit (NZYTech) according to the manufacturer's protocol. AP sites were generated in plasmid pcDNA3.1 (+) by incubating plasmid DNA (163.6 µg/mL) in depurination buffer (10 mM sodium citrate, 100 mM NaCl, pH 5.0) at 70 °C for 20 min. Depurinated DNA was recovered by extraction with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and eluted in TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). DNA quantification was performed by measuring the absorbance at 260 nm, using a SPECTROstar Omega microplate reader in combination with an LVis plate (BMG Labtech, Offenburg, Germany). Depurinated plasmid was aliquoted and stored at -20 °C.

Depurinated plasmid DNA (100 ng) was added to assay reactions containing 0.5 U of human recombinant APE1 in assay buffer (50 mM Tris pH 7.5, 25 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 0.01% Tween-20) and different concentrations of the inhibitor compounds, in a final reaction volume of 18 µL. After an incubation period of 15 min at 37 °C, the reactions were stopped by cooling the solutions on ice and addition of 2 µL of 10× loading buffer (5 PRIME Inc., Gaithersburg, MD, USA), containing 0.21% bromophenol blue, 0.21% xylene cyanol, 20 mM EDTA, pH 8.0, 50% glycerol. The

samples were electrophoresed in 0.8% agarose gel containing GelRed™ (Biotium Inc., Hayward, CA, USA), in 1× Tris-acetate-EDTA buffer at 60 V for 1 h. Assay reactions were prepared in duplicate for each compound concentration and two independent experiments were performed.

4.3.7. Cytotoxicity evaluation of active compounds

The non-tumourigenic human breast epithelial MCF10A cell line was obtained from ATCC (Manassas, VA, USA). MCF10A cells were cultured in monolayer in DMEM/F12 medium supplemented with 5% horse serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.01 mg/mL insulin, 0.5 µg/mL hydrocortisone, 100 ng/mL cholera toxin and 20 ng/mL human epidermal growth factor. Cells were maintained at 37 °C, under a humidified atmosphere containing 5% CO_2 in air.

The effect of APE1 inhibitors on cell viability was evaluated by the MTS tetrazolium assay using the *CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay* kit (Promega, Madison, WI, USA). A 20 mM stock solution of APE1 inhibitors in DMSO was prepared to test compounds at concentrations up to 100 µM. MCF10A cells were seeded at a density of 3×10^3 cells per well, in 200 µL of culture medium, in 96-well plates and incubated for 24 h at 37 °C under a 5% CO_2 atmosphere. Cells were treated with APE1 inhibitors for 48 h. Following the drug treatments, the medium was removed and each well was rinsed with PBS. 20 µL of *CellTiter 96® AQ_{ueous} One Solution Reagent* were added to each well containing 100 µL of culture medium and plates were incubated for 1 h. H_2O_2 (1 mM) was used as a positive control. The final concentration of DMSO in the culture medium was maintained at 0.5% (v/v). The optical density values were obtained at 490 nm in a SPECTROstar Omega microplate reader (BMG Labtech, Offenburg, Germany).

Three independent experiments were performed and three replicate cultures were used for each concentration in each independent experiment. Statistical analysis was performed as described in Chapter 3 (Section 3.3.8).

4.4. RESULTS AND DISCUSSION

4.4.1. SBVS and docking studies

A virtual screening protocol (Figure 4.1) was implemented to identify novel chemical entities from the NCI database showing high affinity for the APE1 domain responsible for the endonuclease activity and aiming to inhibit the BER pathway.

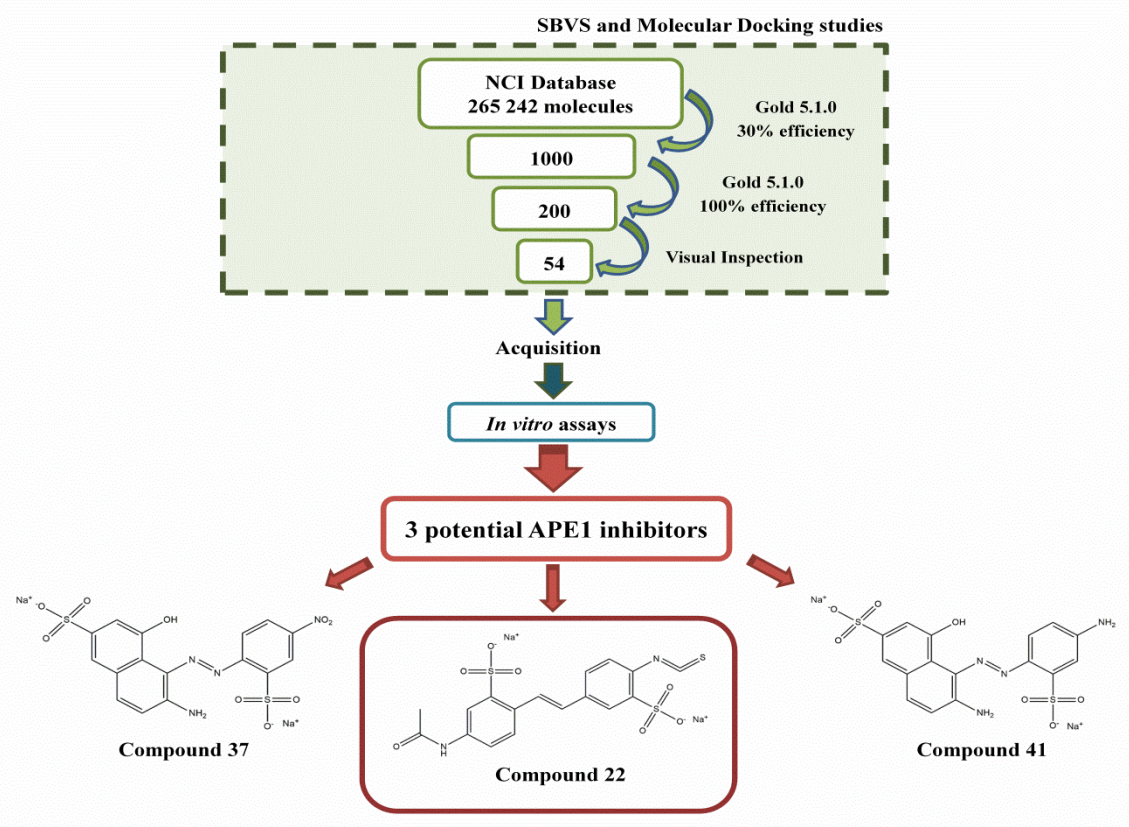


Figure 4.1 Virtual screening protocol and experimental methodologies to identify novel chemical entities from the NCI database showing a high affinity for the APE1 enzyme.

The crystal structure of human APE1 was already resolved. When this work was initiated there were several different 3D structures (PDB codes: 1BIX, 1HD7, 1DE8, 1DEW, 1E9N and 1DE9, for example), complexed with different ligands and having different resolutions (2.2 to 3.0 Å). From all the available structures of APE1, the APE1 X-ray structure deposited in the PDB with the code 1BIX (Figure 4.2A) and a resolution of 2.2 Å [55] was selected for the docking calculations. The metal-binding site identified in this APE1 structure, usually referred to as the "A" site, is in agreement with the binding site of Mg^{2+} or other divalent metals in structures of other members of DNase I superfamily [56,57]. Supported by the similarities amongst the active sites of the available DNA-free human APE1 structures, the PDB 1BIX was chosen in detriment of

the higher resolution PDB entry 1HD7 [58] since it has no missing residues in the catalytic domain and its crystals were grown at a physiological pH of 7.4.

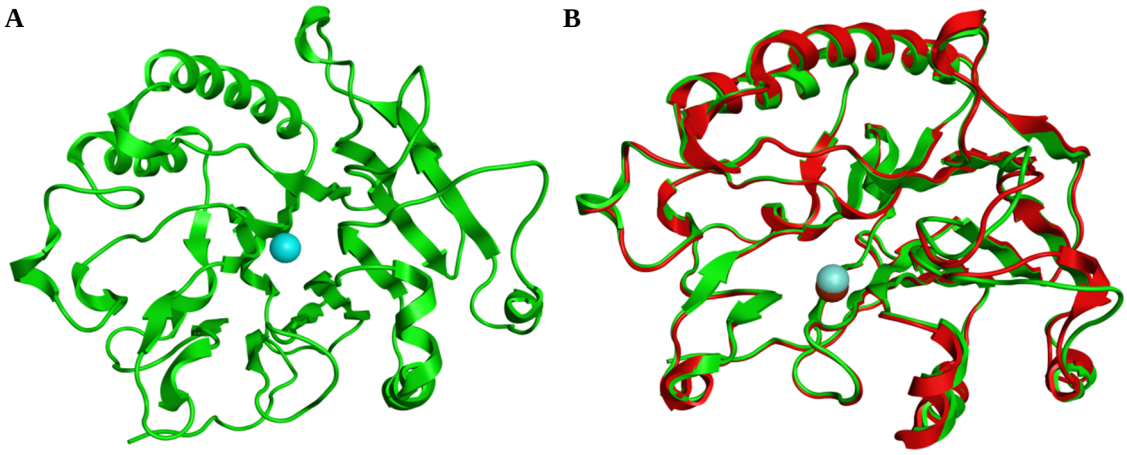


Figure 4.2 APE1 X-ray structures. (A) The crystallographic structure deposited in Protein Data Bank with the code 1BIX (resolution of 2.2 Å) was selected for the docking calculations. (B) Superposition of APE1 X-ray structures with PDB ID: 1BIX (green with the metal in light blue) and PDB ID: 4LND (red with metal in brown), showing a total RMSD of 0.8 Å.

Recently, a new structure of the human APE1 at neutral pH [59] has been solved with a resolution of 1.92 Å. Unfortunately, the latter was not available by the time this work was initiated. Nevertheless, we confirmed that the Mg^{2+} binding site in this new structure, typically referred as the 'A site', is the same as that observed for Sm^{3+} and Pb^{2+} in previous structures of DNA-free APE1 (Figure 4.2B).

These features support the use of the PDB 1BIX [55] and the option for a structure containing a single metal cofactor at the "A" site despite the ongoing controversy on whether the DNase I superfamily enzymes need the presence of two Mg^{2+} (or Ca^{2+}) ions for its catalytic activity [58,60–62] or a single Mg^{2+} cofactor moving from the "A" site in the free enzyme to the "B" site in the enzyme–substrate complex [63].

The initial stages of SBVS of an extensive database such as the one in use are computationally very demanding. In view of this, an approach that optimises the balance between the precision of docking and the computational resources required was used. SBVS is generally performed with speed-up settings with the intent of discarding many compounds in a suitable amount of time while retains only those which better fit the pocket. A preliminary virtual screening was performed using all the 265 242 small-molecules belonging to the NCI database (Figure 4.1) which were docked into the APE1 active site (obtained from the 1BIX crystallographic structure) and ranked using the GoldScore scoring function. From the compounds preliminarily screened for high-affinity with APE1, only the 1000 highest top-ranked compounds

were retained for posterior refinement with exhaustive docking analyses that predict with higher precision the corresponding binding pose and interactions within the receptor binding pocket. These compounds were re-ranked by their optimised performance. The final 200 highest ranked compounds were visually inspected and only the compounds that presented favorable binding conformations, surface complementarity with the enzyme and important interactions with key pocket residues were selected. Fifty-four compounds were acquired to be experimentally tested.

4.4.2. Inhibition of APE1 endonuclease activity

In this work we choose a fluorescence-based APE1 endonuclease activity assay to screen the ability of the fifty-four selected compounds to inhibit the endonuclease activity of the recombinant APE1. This methodology is based on the cleavage by APE1 of a double-stranded DNA carrying an internal AP site mimetic (THF) and termini labeled with 5' TAMRA and a 3' BHQ-2 quencher moiety [35,64] is valuable because is less susceptible to the potential autofluorescence of the compounds. In fact, fluorescence-based assays for the identification of small-molecule inhibitors may be affected by the inherent fluorescence of a given compound. In this case, by adopting an assay with an APE1 substrate detected in the red-shifted spectral region, this interference can be minimized [35,36,64].

Amongst the 54 acquired compounds only 30 compounds were tested (Annex, Table S1). The remaining compounds were excluded due to poor solubility or precipitation in the assay conditions. In this screen, 30 compounds were evaluated at a concentration of 50 µM. Five compounds (16, 22, 37, 41, and 49) showed ≥ 85% of inhibition of APE1 endonuclease activity.

To exclude the possibility of an non-specific inhibition of APE1 endonuclease activity due to an intrinsic ability of the compounds to quench the fluorescence signal of TAMRA, the five positive hits were tested only in the presence of the DNA strand carrying the fluorophore. Although compound 16 clearly inhibited APE1 activity, it also presented a decrease of more than 25% in the fluorescence intensity of the control (data not shown). Therefore this compound was discarded from further analyses. The remaining compounds 22, 37, 41 and 49 were fully analysed in terms of proof-of-structure criteria. Compound 49, which is closely related to compounds 37 and 41 revealed, however, some issues concerning proof-of-structure criteria (^{1}H NMR, HR-ESI-MS) and was therefore excluded. The mass spectrometry data for the remaining compounds 22, 37 and 41 are presented in Annex, Figure S1. The purity of compounds 22, 37 and 41 was 94.0%, 95.4% and 95.1%, respectively, as evaluated by HPLC-

DAD. Therefore, the IC_{50} values were calculated for compounds 22, 37 and 41. Compound 22 is our best hit, showing an IC_{50} of 253 ± 11 nM (Figure 4.3A), a remarkable inhibition of APE1 activity. The other two compounds identified, 37 and 41, showed good inhibitory activities considering the already known APE1 inhibitors, displaying IC_{50} values in the low micromolar range, 7.76 ± 0.44 µM and 8.85 ± 0.48 µM, respectively (Figure 4.3C and 4.3E). Table 4.1 summarizes all this information and compare the obtained docking scores with the respective IC_{50} values.

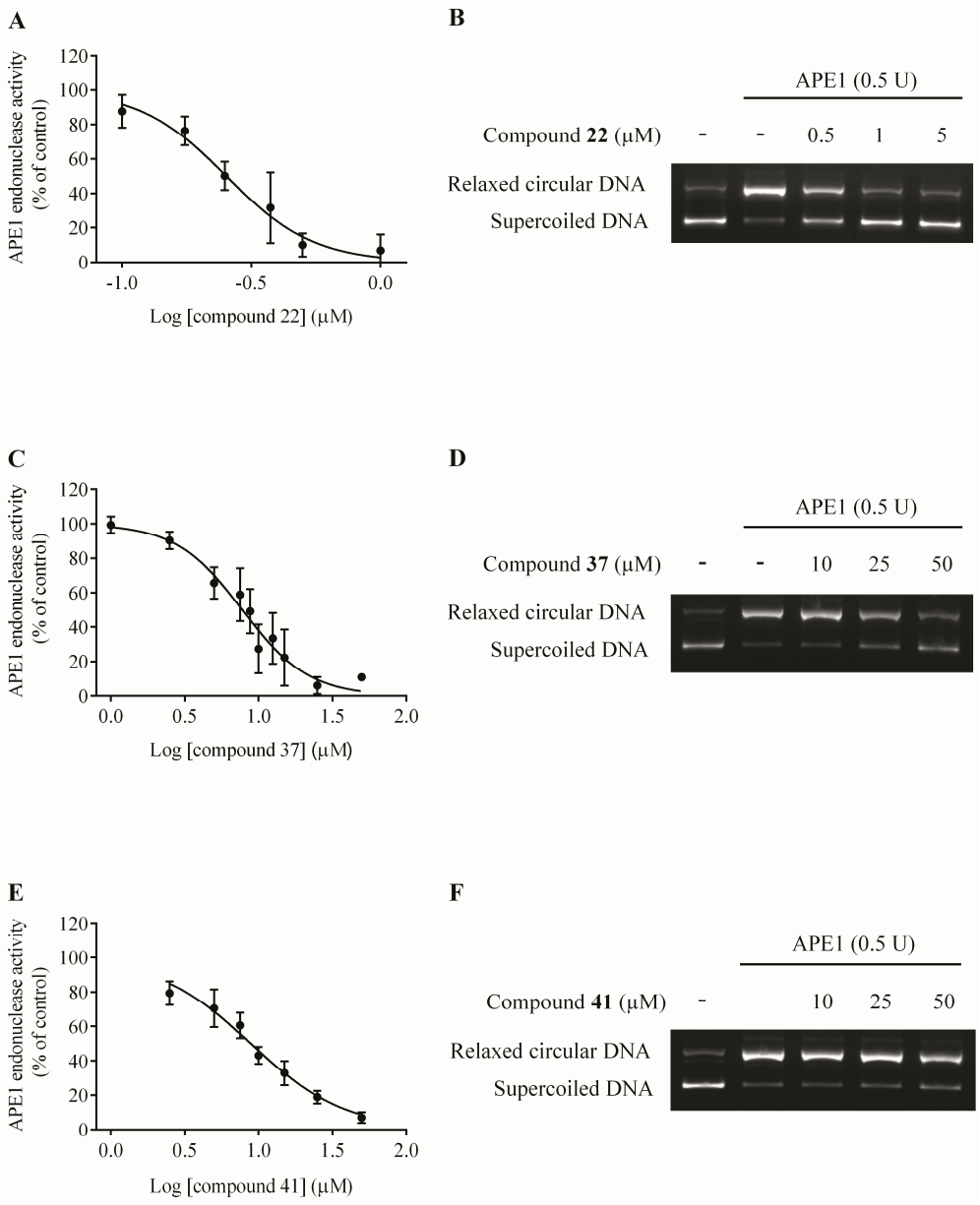
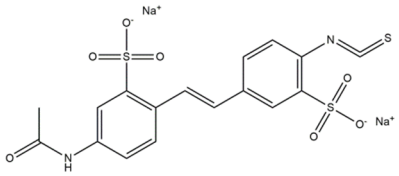
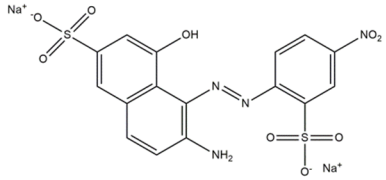
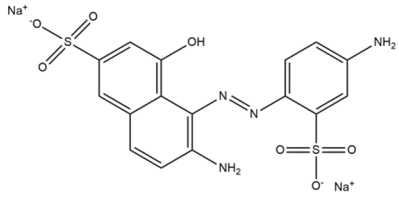


Figure 4.3 Effect of the identified compounds in the APE1 endonuclease activity. The IC_{50} values were obtained from the dose-response curves for compounds (A) 22 (n = 3–7), (C) 37 (n = 2–4) and (E) 41 (n = 3–4) established with a fluorescence-based APE1 endonuclease activity assay. Values represent mean ± SD and are expressed as percentage of activity relative to the negative control. The plasmid nicking assay was performed to confirm the ability of identified compounds to inhibit APE1 endonuclease activity. Representative agarose gels electrophoresis show a decrease in the relaxed circular DNA band in the presence of increasing concentrations of compounds (B) 22, (D) 37 and (F) 41. Two independent experiments were performed in duplicate.

In an attempt to characterize the enzymatic inhibition mode of the most promising compound, we adopted the graphical procedure based on normalised rates (the degree of inhibition) described by Antunes *et al.* [65]. The linear decrease of the degree of inhibition with increasing substrate concentrations in the presence of compound 22 suggests a competitive inhibition mechanism (Annex, Figure S2).

Table 4.1 Structure, lipophilicity (LogP), GoldScore, and IC_{50} values of the identified APE1 inhibitors.

Structure	NSC number	logP[a)]	GoldScore[b)]	IC_{50}[c)] (µM)
 **Compound 22**	NSC 378144	2.85	74.47	0.25 ± 0.01
 **Compound 37**	NSC 47703	1.89	71.73	7.76 ± 0.44
 **Compound 41**	NSC 401610	1.28	71.24	8.85 ± 0.48

[a)] logP (octanol-water partition coefficient) - calculated with MOE 2011.10 software package for the structures used in the the virtual screening protocol.

[b)] GoldScore – value of scoring function for the top pose obtained after 500 GA runs.

[c)] IC_{50} – values determined with the fluorescence-based APE1 endonuclease activity assay.

A counterscreen assay was performed to confirm these findings and validate the compounds as APE1 inhibitors. The plasmid DNA nicking assay is a gel-based assay, where the supercoiled plasmid DNA containing AP sites is converted into the relaxed form in the presence of APE1. The results of this assay showed that the compounds were able to inhibit the enzyme APE1. In fact, compound 22 at 5 µM inhibited completely the enzyme APE1, producing supercoiled and relaxed bands with comparable intensity to that observed for the plasmid DNA in the absence of the

enzyme (Figure 4.3B). Compound 37 also presents a decrease in the intensity of the relaxed band that is dose-dependent and apparently inhibit APE1 endonuclease activity completely at the highest concentration tested (Figure 4.3D). For compound 41 only a slight effect was observed (Figure 4.3F) which means that a higher concentration should be considered. It should be noted that the compounds were tested at higher concentrations in the gel-based assay when compared to the fluorescence-based assay due to methodological differences as well as assay conditions. Besides being used as a qualitative assay, the plasmid nicking assay recapitulated the results of the fluorescence-based assay.

4.4.3. Ligand docking binding modes

An analysis of the best ranked ligand docking poses resulting from the SBVS calculations revealed that all 30 assayed molecules fit well within the APE1 binding site, occupying the pocket defined by the amino acids Asp70, Glu96, Arg177, His309, Asp210, Asn212, Trp280, Phe266, and Leu282, which was quite promising for good ligand-enzyme interactions. Overall, the predicted binding poses highlight in general a close proximity with the metallic cation, hydrogen bonding interactions with the active site residues His309, Asp210, Asn212, Gly231 and Arg177 and a few hydrophobic and π-π interactions of the ligands with Phe266, Trp280 and His309.

A more detailed analysis was performed for the best docking poses of the three active compounds against APE1. In fact, the high docking scores obtained for these compounds, ranging from 75 to 71, already suggested a strong interaction with APE1 when compared with the affinities (docking scores) achieved for lucanthone and hycanthone (scores in the range of 50-55).

Compound 22 (5-(acetylamino)-2-[2-(4-isothiocyanato-3-sulfophenyl)ethenyl] benzenesulfonic acid disodium salt) displays an IC_{50} value consistent with a good inhibitory activity against APE1 *in vitro*. This compound displays structural features which are consistent with the 3D pharmacophore models that were generated by Zawahir *et al.*, based on a set of interactions of the abasic DNA within the APE1 catalytic active site [38]. In that work the most potent and selective inhibitors showing IC_{50} values below 10 μM displayed two terminal negatively-charged groups and a central hydrophobic core in agreement with the arrangement of the 3' and 5' deoxyribose phosphate groups on abasic DNA. In compound 22 the two negatively-charged sulfonate groups (SO_3^{2-}) establish important hydrogen bond interactions with Asn212 (1.6 Å) and Arg177 (1.9 and 2.3 Å) (the interaction with His309 is very weak). This is in accordance with the interactions between the abasic DNA negatively-charged

phosphate group and the APE1 amino acid residues Asn174, Asn212, His309 in the co-crystal structure of the APE1 bound to abasic DNA (PDB code: 1DEW) [66]. This feature is shared by some of the compounds in Table 4.1. Also, compound 22 shows a biphenyl moiety, a group present in some already patented small-molecules inhibitors of APE1 and collected in a recent review by Al-Safi *et al.* [67]. In this review the authors describe all the patented small-molecule inhibitors of APE1 before 2011 and is an excellent starting point to understand the key features present in these compounds. They cover a comprehensive number of compounds with a large chemical diversity, containing molecules with "drug-like" and "non-drug-like" properties. Al-Safi *et al.* [67] describe several chemical structures containing a sulfonamide core showing moderate APE1 inhibitory activity (in the low micromolar range). However, to the best of our knowledge, no patented APE1 inhibitors show the sulfonate group, present in our active compounds. In addition, the presence of the isothiocyanate (R-N=C=S) group in compound 22 provides an additional interesting chemical feature since naturally occurring and synthetic isothiocyanates are emerging anticancer agents [68–70]. In fact, an extra interaction could be established between the terminal sulfur of the isothiocyanate group and Gly231 (at a distance of 3 Å). Interestingly the terminal sulfur is pointing to the center of Trp280 aromatic ring. We suggest that the high potency of compound 22 should result from the coincident presence of the biphenyl core and the sulfonate moieties coupled with a correct position given by the terminal sulfur interactions (Figure 4.4A).

While not as potent as compound 22, compounds 37 (6-amino-4-hydroxy-5-[(4-nitro-2-sulfophenyl)azo]-2-naphtalenesulfonic acid disodium salt) and 41 (6-amino-5-[(4-amino-2-sulfophenyl)azo]-4-hydroxy-2-naphtalenesulfonic acid disodium salt) also revealed significant inhibitory activity. Predicted poses of the active compounds 37 and 41 are shown in Figure 4.4B and 4.4C. These compounds share a very similar pose (nearly overlapped) inside APE1 binding site. In fact these compounds are structurally very similar, only differing on one substituent, $-NO_2$ in the case of compound 37 and $-NH_2$ in the case of compound 41. Again, these compounds have two sulfonate (SO_3^{2-}) groups, which interact with APE1 and establish important hydrogen bonds and ionic interactions. One of the sulfonate groups establishes two hydrogen bonds with His309 (2.7 and 2.8 Å), and the other interacts with Arg177 (2 and 2.2 Å). The benzyl moiety bound to this sulfonate is also able to form an additional weak H-π interaction with Arg177.

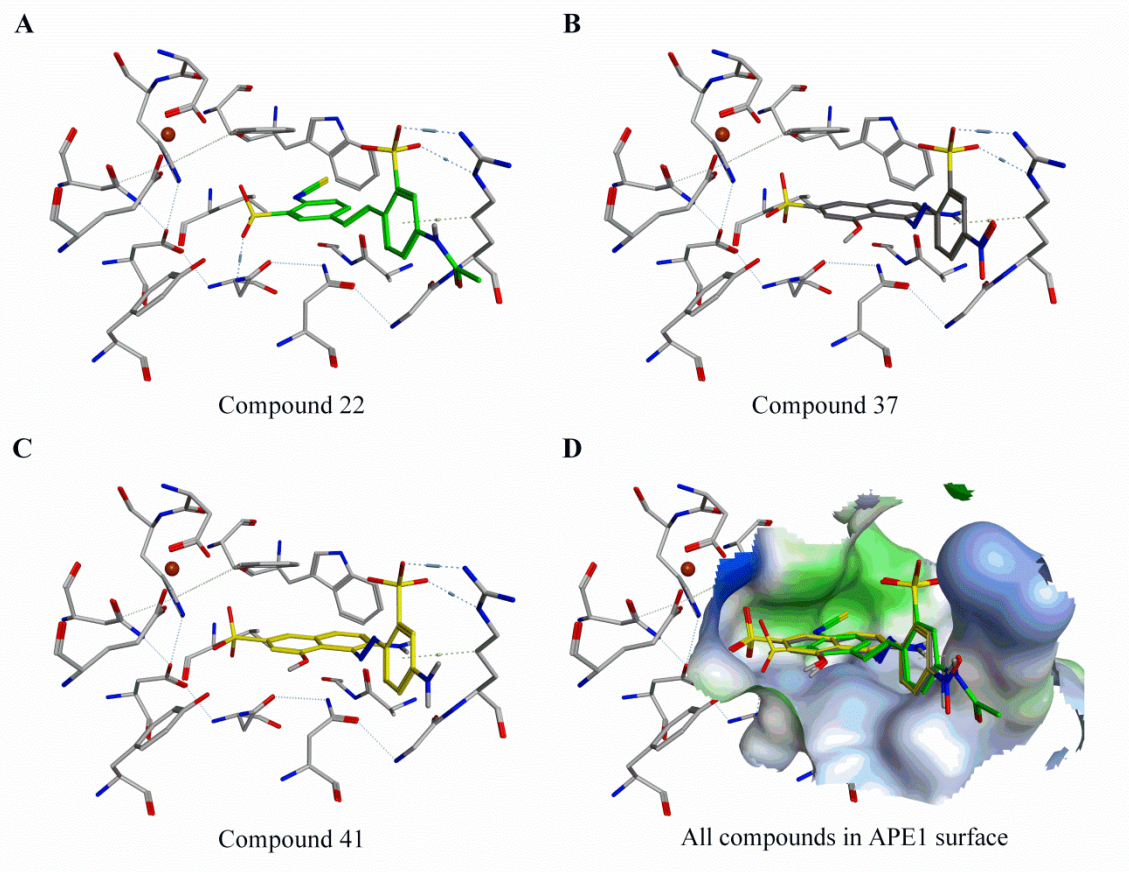


Figure 4.4 Best poses of active compounds inside the APE1 binding pocket.

Overall, these results are in agreement with previously reported studies in the literature by Simeonov *et al.* [35], where compounds resulting from a screen of 1280 compounds from the library of pharmacological active compounds (LOPAC) having sulfonate groups, like Reactive Blue 2 and PPNDS are able to inhibit the APE1 activity. As in our studies, some of the compounds identified demonstrate the importance of negatively ionisable groups for potent APE1 inhibition. By the analysis of the docking poses inside the APE1 binding site we consider that these compounds could be promising scaffolds to optimization to obtain improved APE1 inhibitors.

4.4.4. Evaluation of the cytotoxicity of potential APE1 inhibitors in MCF10A cells

Since APE1 is an ubiquitous protein we cannot exclude the possibility that a decrease in APE1 endonuclease activity may be cytotoxic for tumour but also for non-tumour cells. Therefore, the efficacy and safety of compounds targeting APE1 endonuclease activity can be impaired due to the development of off-target effects. To evaluate the cytotoxicity of the APE1 inhibitors in non-cancer cells, we performed the MTS tetrazolium assay in human mammary epithelial MCF10A cells. After a 48 h exposure

to the compounds at concentrations up to 100 μM, the results show that all the potential APE1 inhibitors are non-toxic to MCF10A cells (Figure 4.5).

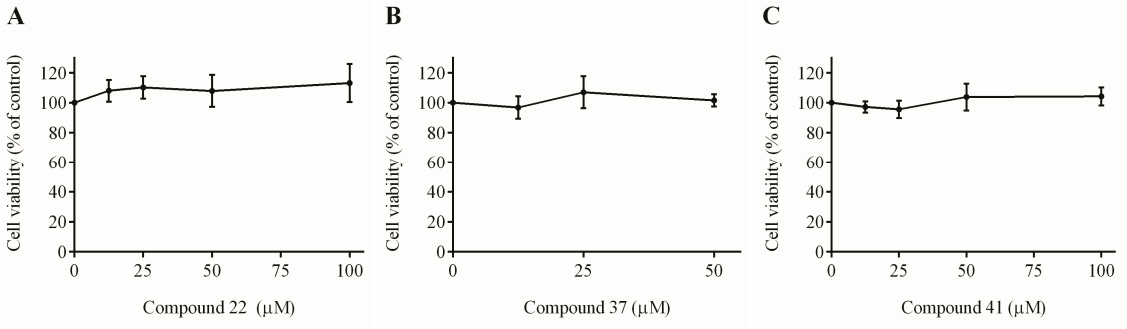


Figure 4.5 Effect of the compounds in the viability of MCF10A cells evaluated by the MTS assay. Cells were incubated with increasing concentrations of compounds (A) 22, (B) 37 and (C) 41 for 48 h. Values represent mean ± SD (n = 3) and are expressed as percentages relative to non-treated control cells.

4.5. CONCLUSIONS

APE1 has emerged as a druggable target with important implications in cancer therapy. In fact, functional studies conducted not only with APE1 inhibitors [6,7] but with the presence of a dominant-negative APE1[71,72] and the decrease of expression by targeting the enzyme with small interfering RNA [73], revealed an enhancement of the cellular sensitivity to anticancer agents.

In the present Chapter, we set out to identify three novel compounds (22, 37 and 41) that show a significant inhibitory activity on APE1 enzyme using a synergic campaign combining computational and experimental methodologies. Importantly, compound 22 showed to be active against APE1 at nanomolar concentrations. In addition, when we compare the properties (e.g. molecular weight, logP, druggability) of the active compounds with the same properties for the already described APE1 inhibitors, we conclude that our compounds present adequate features to be an APE1 inhibitor. Furthermore, compounds 22, 37 and 41 are simple small-molecules with good spatial fitting in APE1 active site and establish important interactions with residues essential for the enzyme endonuclease activity (e.g. Trp280, Asn212, Arg177, His309) without toxicity in non-tumour MCF10A cells. Thus, these compounds have potential to be used as lead compounds for further development of more effective APE1 inhibitors.

4.6. REFERENCES

1. Helleday T, Petermann E, Lundin C, Hodgson B, Sharma RA. DNA repair pathways as targets for cancer therapy. Nat Rev Cancer. 2008;8:193–204.

2. Jackson SP, Bartek J. The DNA-damage response in human biology and disease. Nature. 2009;461:1071–1078.

3. Oliveira NG, Castro M, Rodrigues AS, Gil OM, Toscano-Rico JM, Rueff J. DNA-PK inhibitor wortmannin enhances DNA damage induced by bleomycin in V79 Chinese hamster cells. Teratog Carcinog Mutagen. 2002;22:343–351.

4. Znojek P, Willmore E, Curtin NJ. Preferential potentiation of topoisomerase I poison cytotoxicity by PARP inhibition in S phase. Br J Cancer. 2014;111:1319–1326.

5. Willmore E, de Caux S, Sunter NJ, Tilby MJ, Jackson GH, Austin CA, *et al*. A novel DNA-dependent protein kinase inhibitor, NU7026, potentiates the cytotoxicity of topoisomerase II poisons used in the treatment of leukemia. Blood. 2004;103:4659–4665.

6. Bulgar AD, Weeks LD, Miao Y, Yang S, Xu Y, Guo C, *et al*. Removal of uracil by uracil DNA glycosylase limits pemetrexed cytotoxicity: overriding the limit with methoxyamine to inhibit base excision repair. Cell Death Dis. 2012;3:e252.

7. Fishel ML, He Y, Smith ML, Kelley MR. Manipulation of base excision repair to sensitize ovarian cancer cells to alkylating agent temozolomide. Clin Cancer Res. 2007;13:260–267.

8. Yan T, Seo Y, Schupp JE, Zeng X, Desai AB, Kinsella TJ. Methoxyamine potentiates iododeoxyuridine-induced radiosensitization by altering cell cycle kinetics and enhancing senescence. Mol Cancer Ther. 2006;5:893–902.

9. Veuger SJ, Curtin NJ, Richardson CJ, Smith GCM, Durkacz BW. Radiosensitization and DNA repair inhibition by the combined use of novel inhibitors of DNA-dependent protein kinase and poly(ADP-Ribose) polymerase-1. Cancer Res. 2003;63:6008–6015.

10. Oliveira NG, Castro M, Rodrigues AS, Gonçalves IC, Gil OM, Fernandes AP, Toscano-Rico JM, Rueff J. Wortmannin enhances the induction of micronuclei by low and high LET radiation. Mutagenesis. 2003;18:37–44.

11. Shunkwiler L, Ferris G, Kunos C. Inhibition of poly(ADP-ribose) polymerase enhances radiochemosensitivity in cancers proficient in DNA double-strand break repair. Int J Mol Sci. 2013;14:3773–3785.

12. Taverna P, Hwang HS, Schupp JE, Radivoyevitch T, Session NN, Reddy G, *et al*. Inhibition of base excision repair potentiates iododeoxyuridine-induced cytotoxicity and radiosensitization. Cancer Res. 2003;63:838–846.

13. Rodrigues AS, Gomes BC, Martins C, Gromicho M, Oliveira NG, Guerreiro PS, *et al*. DNA repair and resistance to cancer therapy. In: Chen C, editor. New Research Directions in DNA Repair. InTech; 2013. pp. 489–528.

14. Kelley MR, Logsdon D, Fishel ML. Targeting DNA repair pathways for cancer treatment: what's new? Future Oncol. 2014;10:1215–1237.

15. Abbotts R, Madhusudan S. Human AP endonuclease 1 (APE1): from mechanistic insights to druggable target in cancer. Cancer Treat Rev. 2010;36:425–435.

16. Guerreiro PS, Fernandes AS, Costa JG, Castro M, Miranda JP, Oliveira NG. Differential effects of methoxyamine on doxorubicin cytotoxicity and genotoxicity in MDA-MB-231 human breast cancer cells. Mutat Res. 2013;757:140–147.

17. Kelley MR, Georgiadis MM, Fishel ML. APE1/Ref-1 role in redox signaling: translational applications of targeting the redox function of the DNA repair/redox protein APE1/Ref-1. Curr Mol Pharmacol. 2012;5:36–53.

18. Bobola MS, Blank A, Berger MS, Stevens BA, Silber JR. Apurinic/Apyrimidinic Endonuclease Activity Is Elevated in Human Adult Gliomas. Clin Cancer Res. 2001;7:3510–3518.

19. Di Maso V, Avellini C, Crocè LS, Rosso N, Quadrifoglio F, Cesaratto L, *et al*. Subcellular localization of APE1/Ref-1 in human hepatocellular carcinoma: possible prognostic significance. Mol Med. 2007;13:89–96.

20. Kakolyris S, Kaklamanis L, Engels K, Turley H, Hickson ID, Gatter KC, *et al*. Human Apurinic Endonuclease 1 expression in a colorectal adenoma-carcinoma sequence. Cancer Res. 1997;57:1794–1797.

21. Kelley MR, Cheng L, Foster R, Tritt R, Jiang J, Broshears J, *et al*. Elevated and altered expression of the multifunctional DNA base excision repair and redox enzyme Ape1/ref-1 in prostate cancer. Clin Cancer Res. 2001;7:824–830.

22. Kakolyris S, Kaklamanis L, Engels K, Fox SB, Taylor M, Hickson ID, *et al*. Human AP endonuclease 1 (HAP1) protein expression in breast cancer correlates with lymph node status and angiogenesis. Br J Cancer. 1998;77:1169–1173.

23. Wang D, Luo M, Kelley MR. Human apurinic endonuclease 1 (APE1) expression and prognostic significance in osteosarcoma: Enhanced sensitivity of osteosarcoma to DNA damaging agents using silencing RNA APE1 expression inhibition. Mol Cancer Ther. 2004;3:679–686.

24. Puglisi F, Aprile G, Minisini AM, Barbone F, Cataldi P, Tell G, *et al*. Prognostic significance of Ape1/ref-1 subcellular localization in non-small cell lung carcinomas. Anticancer Res. 2001;21:4041–4049.

25. Liuzzi M, Talpaert-Borle M. A new approach to the study of the base-excision repair pathway using methoxyamine. J Biol Chem. 1985;260:5252–5258.

26. Yan L, Bulgar A, Miao Y, Mahajan V, Donze JR, Gerson SL, *et al*. Combined treatment with temozolomide and methoxyamine: blocking apurininc/pyrimidinic site repair coupled with targeting topoisomerase IIalpha. Clin Cancer Res. 2007;13:1532–1539.

27. Madhusudan S, Smart F, Shrimpton P, Parsons JL, Gardiner L, Houlbrook S, *et al*. Isolation of a small molecule inhibitor of DNA base excision repair. Nucleic Acids Res. 2005;33:4711–4724.

28. Mohammed MZ, Vyjayanti VN, Laughton CA, Dekker L V, Fischer PM, Wilson DM 3rd, *et al*. Development and evaluation of human AP endonuclease inhibitors in melanoma and glioma cell lines. Br J Cancer. 2011;104:653–663.

29. Bapat A, Glass LS, Luo M, Fishel ML, Reed A, Long EC, *et al*. Novel small-molecule inhibitor of apurinic/apyrimidinic endonuclease 1 blocks proliferation and reduces viability of glioblastoma cells. J Pharmacol Exp Ther. 2010;334:988–998.

30. Liu L, Yan L, Donze JR, Gerson SL. Blockage of abasic site repair enhances antitumor efficacy of 1,3-bis-(2-chloroethyl)-1-nitrosourea in colon tumor xenografts. Mol Cancer Ther. 2003;2:1061–1066.

31. Bapat A, Fishel ML, Kelley MR. Going ape as an approach to cancer therapeutics. Antioxid Redox Signal. 2009;11:651–668.

32. Naidu MD, Agarwal R, Pena LA, Cunha L, Mezei M, Shen M, *et al*. Lucanthone and its derivative hycanthone inhibit apurinic endonuclease-1 (APE1) by direct protein binding. PLoS One. 2011;6:e23679.

33. Luo M, Kelley MR. Inhibition of the human apurinic/apyrimidinic endonuclease (APE1) repair activity and sensitization of breast cancer cells to DNA alkylating agents with lucanthone. Anticancer Res. 2004;24:2127–2134.

34. Seiple LA, Cardellina JH, Akee R, Stivers JT. Potent inhibition of human apurinic/apyrimidinic endonuclease 1 by arylstibonic acids. Mol Pharmacol. 2008;73:669–677.

35. Simeonov A, Kulkarni A, Dorjsuren D, Jadhav A, Shen M, McNeill DR, *et al*. Identification and characterization of inhibitors of human apurinic/apyrimidinic endonuclease APE1. PLoS One. 2009;4:e5740.

36. Dorjsuren D, Kim D, Vyjayanti VN, Maloney DJ, Jadhav A, Wilson DM 3rd, *et al*. Diverse small molecule inhibitors of human apurinic/apyrimidinic endonuclease APE1 identified from a screen of a large public collection. PLoS One. 2012;7:e47974.

37. Rai G, Vyjayanti VN, Dorjsuren D, Simeonov A, Jadhav A, Wilson DM 3rd, *et al*. Synthesis, biological evaluation, and structure–activity relationships of a novel class of apurinic/apyrimidinic endonuclease 1 inhibitors. J Med Chem. 2012;55:3101–3112.

38. Zawahir Z, Dayam R, Deng J, Pereira C, Neamati N. Pharmacophore guided discovery of small-molecule human apurinic/apyrimidinic endonuclease 1 inhibitors. J Med Chem. 2009;52:20–32.

39. Aiello F, Shabaik Y, Esqueda A, Sanchez TW, Grande F, Garofalo A, *et al*. Design and synthesis of 3-carbamoylbenzoic acid derivatives as inhibitors of human apurinic/apyrimidinic endonuclease 1 (APE1). ChemMedChem. 2012;7:1825–1839.

40. Feng Z, Kochanek S, Close D, Wang L, Srinivasan A, Almehizia AA, *et al*. Design and activity of AP endonuclease-1 inhibitors. J Chem Biol. 2015;8:79–93.

41. Srinivasan A, Wang L, Cline CJ, Xie Z, Sobol RW, Xie XQ, *et al*. Identification and characterization of human apurinic/apyrimidinic endonuclease-1 inhibitors. Biochemistry. 2012;51:6246–6259.

42. Ruiz FM, Francis SM, Tintoré M, Ferreira R, Gil-Redondo R, Morreale A, *et a*l. Receptor-based virtual screening and biological characterization of human apurinic/apyrimidinic endonuclease (Ape1) inhibitors. ChemMedChem. 2012;7:2168–2178.

43. Jorgensen WL. The many roles of computation in drug discovery. Science. 2004;303:1813–1818.

44. Cavasotto C, W. Orry A. Ligand docking and dtructure-based virtual screening in drug discovery. Curr Top Med Chem. 2007;7:1006–1014.

45. Ghosh S, Nie A, An J, Huang Z. Structure-based virtual screening of chemical libraries for drug discovery. Curr Opin Chem Biol. 2006;10:194–202.

46. Eckert H, Bajorath J. Molecular similarity analysis in virtual screening: foundations, limitations and novel approaches. Drug Discov Today. 2007;12:225–233.

47. Ripphausen P, Nisius B, Peltason L, Bajorath J. Quo vadis, virtual screening? A comprehensive survey of prospective applications. J Med Chem. 2010;53:8461–8467.

48. Jones G, Willett P, Glen RC. Molecular recognition of receptor sites using a genetic algorithm with a description of desolvation. J Mol Biol. 1995;245:43–53.

49. Jones G, Willett P, Glen RC, Leach AR, Taylor R. Development and validation of a genetic algorithm for flexible docking. J Mol Biol. 1997;267:727–748.

50. Molecular Operating Environment (MOE), 2011.10; Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, 2011.

51. Verdonk ML, Cole JC, Hartshorn MJ, Murray CW, Taylor RD. Improved protein-ligand docking using GOLD. Proteins. 2003;52:609–623.

52. Wang J, Cieplak P, Kollman PA. How well does a restrained electrostatic potential (RESP) model perform in calculating conformational energies of organic and biological molecules? J Comput Chem. 2000;21:1049–1074.

53. Lowry DF, Hoyt DW, Khazi FA, Bagu J, Lindsey AG, Wilson DM 3rd. Investigation of the Role of the Histidine–Aspartate Pair in the Human Exonuclease III-like Abasic Endonuclease, Ape1. J Mol Biol. 2003;329:311–322.

54. Mendez F, Goldman JD, Bases RE. Abasic sites in DNA of HeLa cells induced by lucanthone. Cancer Invest. 2002;20:983–991.

55. Gorman MA, Morera S, Rothwell DG, de La Fortelle E, Mol CD, Tainer JA, *et al*. The crystal structure of the human DNA repair endonuclease HAP1 suggests the recognition of extra-helical deoxyribose at DNA abasic sites. EMBO J. 1997;16:6548–6558.

56. Suck D, Oefner C. Structure of DNase I at 2.0 Å resolution suggests a mechanism for binding to and cutting DNA. Nature. 1986;321:620–625.

57. Dlakić M. Functionally unrelated signalling proteins contain a fold similar to Mg^{2+}-dependent endonucleases. Trends Biochem Sci. 2000;25:272–273.

58. Beernink PT, Segelke BW, Hadi MZ, Erzberger JP, Wilson DM 3rd, Rupp B. Two divalent metal ions in the active site of a new crystal form of human apurinic/apyrimidinic endonuclease, Ape1: implications for the catalytic mechanism. J Mol Biol. 2001;307:1023–1034.

59. Manvilla BA, Wauchope O, Seley-Radtke KL, Drohat AC. NMR studies reveal an unexpected binding site for a redox inhibitor of AP endonuclease 1. Biochemistry. 2011;50:10540–10549.

60. Jones SJ, Worrall AF, Connolly BA. Site-directed mutagenesis of the catalytic residues of bovine pancreatic deoxyribonuclease I. J Mol Biol. 1996;264:1154–1163.

61. Tsutakawa SE, Shin DS, Mol CD, Izumi T, Arvai AS, Mantha AK, *et al*. Conserved structural chemistry for incision activity in structurally non-homologous apurinic/apyrimidinic endonuclease APE1 and endonuclease IV DNA repair enzymes. J Biol Chem. 2013;288: 8445–8455.

62. Manvilla BA, Pozharski E, Toth EA, Drohat AC. Structure of human apurinic/apyrimidinic endonuclease 1 with the essential Mg^{2+} cofactor. Acta Crystallogr D Biol Crystallogr. 2013;69:2555–2562.

63. Oezguen N, Schein CH, Peddi SR, Power TD, Izumi T, Braun W. A “moving metal mechanism” for substrate cleavage by the DNA repair endonuclease APE-1. Proteins. 2007;68:313–323.

64. Simeonov A, Jadhav A, Thomas CJ, Wang Y, Huang R, Southall NT, *et al*. Fluorescence spectroscopic profiling of compound libraries. J Med Chem. 2008;51:2363–2371.

65. Antunes F, Marinho HS, Barreto MC, Pavão ML, Pinto RE. Diagnosis of enzyme inhibition based on the degree of inhibition. Biochim Biophys Acta. 2003;1624:11–20.

66. Mol CD, Izumi T, Mitra S, Tainer JA. DNA-bound structures and mutants reveal abasic DNA binding by APE1 and DNA repair coordination. Nature. 2000;403:451–456.

67. Al-Safi RI, Odde S, Shabaik Y, Neamati N. Small-molecule inhibitors of APE1 DNA repair function: an overview. Curr Mol Pharmacol. 2012;5:14–35.

68. Wu X, Zhou Q, Xu K. Are isothiocyanates potential anti-cancer drugs? Acta Pharmacol Sin. 2009;30:501–512.

69. Minarini A, Milelli A, Fimognari C, Simoni E, Turrini E, Tumiatti V. Exploring the effects of isothiocyanates on chemotherapeutic drugs. Expert Opin Drug Metab Toxicol. 2014;10:25-38.

70. Jakubikova J, Cervi D, Ooi M, Kim K, Nahar S, Klippel S, *et al*. Anti-tumor activity and signaling events triggered by the isothiocyanates, sulforaphane and phenethyl isothiocyanate, in multiple myeloma. Haematologica. 2011;96:1170–1179.

71. McNeill DR, Wilson DM 3rd. A dominant-negative form of the major human abasic endonuclease enhances cellular sensitivity to laboratory and clinical DNA-damaging agents. Mol Cancer Res. 2007;5:61–70.

72. McNeill DR, Lam W, DeWeese TL, Cheng YC, Wilson DM 3rd. Impairment of APE1 function enhances cellular sensitivity to clinically relevant alkylators and antimetabolites. Mol Cancer Res. 2009;7:897–906.

73. Fung H, Demple B. Distinct roles of Ape1 protein in the repair of DNA damage induced by ionizing radiation or bleomycin. J Biol Chem. 2011;286:4968–4977.

CHAPTER 5

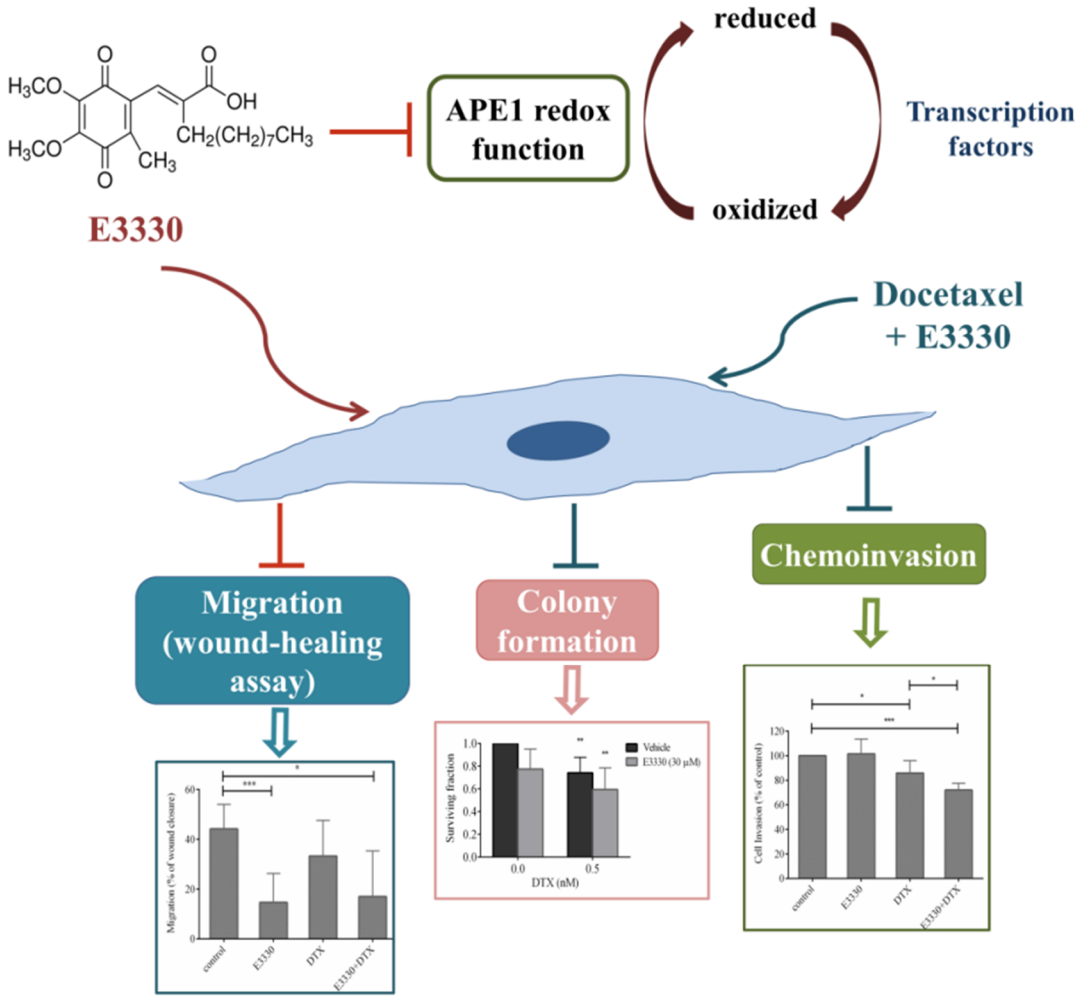
THE APE1 REDOX INHIBITOR E3330 REDUCES COLLECTIVE CELL MIGRATION OF HUMAN BREAST CANCER CELLS AND DECREASES CHEMOINVASION AND COLONY FORMATION WHEN COMBINED WITH DOCETAXEL

This Chapter was adapted from:

Guerreiro PS, Corvacho E, Costa JG, Saraiva N, Fernandes AS, Castro M, Miranda JP, Oliveira NG. The APE1 redox inhibitor E3330 reduces collective cell migration of human breast cancer cells decreases chemoinvasion and colony formation when combined with docetaxel. Chem Biol Drug Des. (submitted).

5.1. ABSTRACT

APE1 is an ubiquitous multifunctional DNA repair enzyme and a redox signalling protein. Our work addressed the inhibition of APE1 redox function using E3330, as single agent or in combination with DTX, in human breast cancer MDA-MB-231 cells. E3330 decreased the colony formation of DTX-treated cells. In addition, E3330 alone significantly reduced the collective cell migration as assessed by the wound healing assay whereas the combined treatment decreased chemoinvasion. These results suggest that the inhibition of APE1 redox function might have therapeutic potential by modulating cell migration and invasion in metastatic breast cancer.



Keywords

Apurinic/apyrimidinic endonuclease 1, Breast cancer, Docetaxel, E3330, Cell migration and invasion

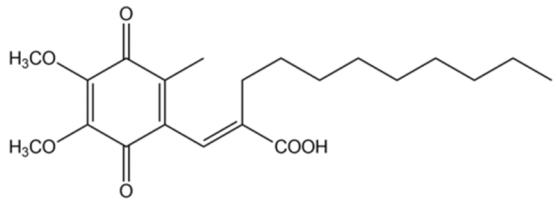
5.2. INTRODUCTION

Breast cancer is the most common cancer diagnosed worldwide in women [1,2]. Despite the improvement in early diagnosis and the advances in adjuvant therapy, it remains one of the leading causes of tumour-related death [1–3]. The mortality of breast cancer is mainly caused by the development of metastases at distant sites from the primary tumour [3–5]. In addition to the clinical heterogeneity of primary tumours, the heterogeneous metastatic pattern usually impairs the efficacy of the current treatments contributing to the poor prognosis of advanced breast cancer [3–5].

APE1 is an ubiquitous multifunctional protein of BER pathway. The endonuclease activity is the major DNA repair function of APE1 and it is essential for the recognition and processing of AP sites generated by exogenous and/or endogenous agents participating in the maintenance of the genome stability. APE1 has also other DNA repair functions which include 3'-phosphodiesterase, weak 3'-phosphatase, 3'-5'exonuclease and RNase H activity [6,7]. APE1 is usually referred as a dual function protein because it has also an independent role as a reduction/oxidation signalling protein, modulating the activation of several transcription factors through the reduction of the cysteine residues in their DNA binding domains. Consequently, APE1 regulates the ability of transcription factors, such as NF-κB [8], AP-1 [9], Egr-1 [10,11], HIF-1α [12–14], p53 [15,16], STAT3 [17] among others, to bind to their specific DNA sequence and promote the expression of genes implicated in cancer cell survival, proliferation, migration/invasion, angiogenesis and metastases formation [6,7]. More recently, the redox function of APE1 has been shown to be involved in the regulation of WNT/β-catenin signalling pathway in pancreatic cancer cells [18]. Furthermore, alterations in the expression and subcellular distribution of APE1 have been reported in numerous types of tumours [19,20], including breast cancer [21–23].

APE1 has gained increasing attention as an emerging drug target in cancer therapy. The DNA repair function has been the focus for the development of several novel small-molecule inhibitors in an attempt to address the resistance to current anticancer therapies and to improve the clinical outcomes by increasing cancer cell death [20,24]. In addition, the plethora of transcription factors modulated by APE1 redox function and their roles in a variety of cellular processes render APE1 as an important upstream effector in several diseases including cancer [7]. In fact, the modulation of APE1 redox function might simultaneously impair several pathways required for cancer development and progression.

E3330 (Fig.5.1) is a quinone derivative already described as a direct inhibitor of the redox function of APE1 [7,25] which has been used in different cell-based [14,26–28] and *in vivo* [29,30] assays revealing its therapeutic potential. The nature of E3330-APE1 interaction has been widely studied to unveil the mechanism of the inhibition of APE1's redox activity. Su *et al.* [31] proposed that E3330 binds to a partially unfolded conformation of APE1 stabilizing the protein and allowing the exposure of buried Cys residues critical for the redox activity which become available to reduce the transcription factors. Moreover, E3330 may facilitate the disulfide bond formation probably between the residues Cys65 and Cys93 decreasing the APE1 redox active molecules [31]. Using a NMR shift assay and docking studies, Manvilla *et al.* [32] suggested that the E3330 binding site in APE1 is located in the vicinity of the DNA repair active site. In this case E3330 could act as an allosteric inhibitor suppressing the conformational change of APE1 required for its redox activity and/ or disrupting the binding or activation of transcription factors [32]. More recently, the findings of Zhang *et al.* [33] were consistent with the E3330-APE1 binding mechanism proposed by Su *et al.* [31] supporting the binding of the redox inhibitor to a partially unfolded state of APE1 to inhibit its redox function.



E3330

Figure 5.1 Chemical structure of the APE1 redox inhibitor (2E)-2-[(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)methylene] undecanoic acid (E3330).

E3330 was found to suppress the inflammatory response of activated macrophages [26] and to prevent the induction of inflammatory cytokines (IL-8 and IL-6) expression in hepatic cancer cell lines [34] suggesting that this inhibitor can be used in inflammatory processes associated with tumours and liver pathologies. E3330 also inhibited the differentiation of adult human bone-marrow-derived mesenchymal stem cells (BM-MSCs) and the growth of endothelial cell precursors and pancreatic cancer-associated endothelial cells [28]. Moreover, E3330 also impaired the migration and the endothelial cell tube formation of retinal vascular endothelial cells isolated from mice [30] as well as mitigated the progression of choroidal neovascularization in mouse eyes, supporting its role in the inhibition of angiogenesis [35,36]. In addition, E3330 has been shown to decrease the growth [14,17,18,29] and migration of human pancreatic cancer cells in *in*

vitro models [14]. The evaluation of this compound in a pancreatic tumour xenograft model revealed a decrease of the tumours growth rate and promising pharmacokinetics and pharmacodynamics properties [29]. Overall, these evidences suggest the usefulness of E3330 as a bioactive compound to reduce tumour invasion and metastatic disease in other unexploited cancer models. In this context, in the present Chapter we addressed this topic and performed the assessment of the impact of a novel therapeutic strategy based on targeting APE1 redox function with E3330 in human breast cancer MDA-MB-231 cells. In this innovative approach the effect of E3330 *per se* or in combination with DTX, a widely used taxane in breast cancer chemotherapy, were thoroughly evaluated. In fact, complementary endpoints were used to assess cell viability, colony formation and cell cycle distribution profile. Since the ability of cells to migrate and invade the surrounding tissues is essential for the development of metastases, both anti-migratory and anti-invasive properties were explored, revealing important findings either for E3330 as single agent or in combination with DTX.

5.3. MATERIALS AND METHODS

5.3.1. Chemicals

DMEM, PBS (0.01 M, pH 7.4), crystal violet (CV), glutaraldehyde, DMSO, RNase A, DTX (purity ≥ 97%), E3330 (purity ≥ 98%) were purchased from Sigma-Aldrich (Madrid, Spain). A 2 mM stock solution of DTX was prepared in DMSO, aliquoted and stored at -20 °C. E3330 was dissolved in DMSO at 10 mM, aliquoted and stored at -20 °C. FBS, penicillin-streptomycin solution and trypsin/ethylenediaminetetraacetic acid solution were acquired from Gibco (Life Technologies, Madrid, Spain). Ethanol, acetic acid and propidium iodide (PI) were obtained from Merck (Darmstadt, Germany). Human recombinant APE1 was purchased from New England Biolabs (Ipswich, MA, USA). The 17-mer oligonucleotides 5' TAMRA-TC ACC *TC GTA CGA CTC-3' and 3' BHQ-2-AG TGG GAG CAT GCT GAG (in which TAMRA is the fluorophore carboxytetramethylrhodamine, BHQ-2 is black hole quencher-2 and * is tetrahydrofuran, a stable abasic site analogue) [37] were custom-made by NZYTech (Lisbon, Portugal). Both oligonucleotides were dissolved in TE buffer (1 mM EDTA, 10 mM Tris-HCl pH 8.0) at a concentration of 100 µM, aliquoted and stored at -20 °C. Matrigel™ was purchased from BD Biosciences (San Jose, CA, USA).

5.3.2. Cell culture

The human mammary adenocarcinoma MDA-MB-231 cell line (HTB-26) was acquired from American Type Culture Collection (ATCC, Manassa, VA, USA). MDA-MB-231 cells were cultured in monolayer in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 0.1 mg/mL streptomycin, at 37 °C in a humidified atmosphere containing 5% CO_2 in air.

5.3.3. Fluorescence-based APE1 endonuclease activity assay

APE1 endonuclease activity in the presence of E3330 was evaluated with a fluorescence-based assay previously described by Simeonov *et al.* [37] with modifications. This assay was performed according to the procedure described in Chapter 4 (Section 4.3.4). Briefly, E333o was added to the reaction mixtures containing 2.5 U of human recombinant APE1 in assay buffer. After 15 min incubation at room temperature, the reaction was initiated by the addition of the double-stranded DNA substrate to a final concentration of 250 nM. Fluorescence was measured at 1 min intervals for 30 min with incubation at 37 °C using a SpectraMax Gemini™ EM microplate plate reader (Molecular Devices, Berkshire, UK) in the kinetic mode at

excitation wavelength 550 nm, emission wavelength 584 nm and cut-off at 570 nm. The reaction of APE1 in the presence of DMSO 0.5% (v/v) was considered to represent 100% of activity (negative control). APE1 activity in the presence of E3330 (30 or 50 μM) was calculated relatively to the negative control. The arylstibonic acid NSC 13755 was used as a positive control for APE1 endonuclease activity inhibition. Each value is presented as mean ± SD of three independent experiments performed in duplicate.

5.3.4. Crystal violet (CV) staining assay

The effect of DTX alone (0.5–1000 nM) or in the presence of E3330 (30 μM) in the MDA-MB-231 cells viability was first evaluated using the CV staining assay. Cells were seeded at a density of approximately 7×10^3 cells per well, in 200 μL of culture medium, in 96-well plates and incubated for 22 h. MDA-MB-231 cells were treated with E3330 (30 μM) for 2 h. Following the pre-incubation period, DTX (0.5 or 100 nM) was added and MDA-MB-231 cells were incubated for 24 h in the presence of both compounds. The final concentration of DMSO in the culture medium did not exceed 0.5% (v/v). The CV assay was then performed according to a previously described protocol [38,39]. Absorbance values presented by MDA-MB-231 cells exposed to 0.5% DMSO (v/v) alone (vehicle-treated control cultures) corresponded to 100% cell viability. Three to seven independent experiments were carried out and eight replicate cultures were used for each condition in each independent experiment.

5.3.5. MTS reduction assay

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) reduction assay was carried out as a confirmatory assay to evaluate cell viability. Briefly, MDA-MB-231 cells were cultured at a density of approximately 6×10^3 cells per well, in 200 μL of culture medium, in 96-well plates and incubated for 22 h. Cells were treated with DTX and/or E3330 according to the aforementioned protocol for the CV staining assay. Following the drug treatments, the assay was performed as described in Chapter 4 (Section 4.3.7). The absorbance values vehicle-treated control cultures measured at 490 nm corresponded to 100% cell viability. Two to three independent experiments were performed in triplicate.

5.3.6. Colony formation assay

The colony formation assay was performed according to a previously described procedure [20]. MDA-MB-231 cells were seeded into 6-well plates at a cell density ranging from 1×10^2 to 1×10^4 cells per well and maintained in a humidified incubator for 16 h. Cells were exposed to E3330 (30 μM) for 2 h before the addition of DTX (0.5

or 10 nM). After a 24 h incubation period with compounds, the cell cultures were washed with PBS and incubated in drug-free medium for 10 to 12 days to form colonies. Afterwards the assay was performed as described in Chapter 3 (Section 3.3.5). Colonies greater than or equal to 50 cells were visually identified and counted by two investigators. The surviving fraction after drug exposure was normalised to the plating efficiency of vehicle-treated control cells. The drug treatments were evaluated in duplicate and four independent experiments were performed.

5.3.7. Cell DNA content analysis

For cell DNA content analysis, approximately 2.75 × 10^5 MDA-MB-231 cells were plated in 6-well plates and cultured for 22 h. Afterwards, cells were pre-incubated with E3330 (30 µM) for 2 h. DTX (0.5 or 100 nM) was then added and cells were cultured for a further 24 h period in the presence of both drugs. Cells were harvested using 5 mM EDTA in PBS at 37 °C, washed with cold PBS and fixed with chilled 80% ethanol. Cells were stained with propidium iodide (PI; 10 µg/mL) in the presence of RNase A (20 µg/mL) for 15–20 min and were analysed using a FACSCalibur flow cytometer (BD Biosciences) [40]. Data were acquired with CellQuest® software (BD Biosciences) and analysed with FlowJo® X.0.7 (Tree Star Inc., San Carlos, CA, USA). At least three independent experiments were performed.

5.3.8. *In vitro* wound healing assay

The *in vitro* wound healing assay was performed using a previously described protocol [41]. MDA-MB-231 cells were cultured in 24-well plates at a seeding density of 2.75 × 10^5 cells per well. Cells were incubated in complete growth medium for 22 h. The culture medium was removed and a scratch was executed on the confluent cell monolayer with a 200 µL sterile pipette tip resulting in a gap of approximately 0.6–0.7 mm in width. Cells were immediately rinsed twice with serum-free DMEM to remove the detached cells and were cultured in serum-free DMEM in the presence of E3330 (30 µM) and/or DTX (0.5 nM) for 24 h. Wound closure was evaluated microscopically with a Motic AE2000 inverted microscope. Photographs of the same areas of the scratch were taken at an amplification of 40x using a Moticam 2500. The scratches width was measured using Motic Images Plus version 2.0 software at different time points, namely 0, 8 and 24 h post-scratch and compounds addition. Cellular motility was analysed in relation to the initial distance between the two scratches edges which was considered as 0% of wound closure. Each condition was evaluated in triplicates and four to six independent experiments were performed.

5.3.9. Chemotaxis and chemoinvasion assays

The chemotactic migration and chemoinvasion of MDA-MB-231 cells was evaluated by adopting a protocol already described [41]. The chemotaxis was assessed in 24-well plates by seeding 1×10^5 cells in serum-free medium on the top of a transwell insert with transparent PET membranes containing 8 µm pores (BD Biosciences, USA). Culture media containing 10% FBS as chemoattractant was added to the lower compartment. Compounds were added to both chambers and cells were allowed to migrate through the membrane for 24 h. After the incubation period, non-migrating cells were gently removed from the upper compartment with a cotton swab. Migrated cells present in the bottom of each membrane were fixed with cold 96% ethanol for 15 min and stained with 0.1% crystal violet in 10% ethanol for 10 min. The inserts were rinsed with water and the remaining cell-attached dye was resuspended in 96% ethanol with 1% acetic acid. Absorbance was measured at 595 nm with a SPECTROstar Omega microplate reader (BMG Labtech, Offenburg, Germany). The results were expressed as percentages of vehicle-treated control cells. Five independent experiments were performed.

A procedure similar to the aforementioned for the chemotactic migration assay was used for the assessment of chemoinvasion. In this case, the porous membranes of the transwell inserts were coated with 50 µL of Matrigel™ at a concentration of 0.3 mg/mL in serum-free medium. Following the compounds addition, MDA-MB-231 cells were incubated for 16 h. Five independent experiments were performed.

5.3.10. Statistical analysis

The values presented correspond to mean values and respective standard deviations (SD). Statistical analysis was performed as described in Chapter 3 (Section 3.3.8).

5.4. RESULTS

5.4.1. APE1 endonuclease activity in the presence of E3330

In order to assure that the E3330 effects observed in this work were due to the modulation of APE1 redox function and not due to the inhibition of its DNA repair function, a fluorescence-based endonuclease activity assay previously described [37] was performed. In this work, none of the E3330 tested concentrations (30 and 50 μM) inhibited APE1 endonuclease activity under these conditions (Fig. 5.2).

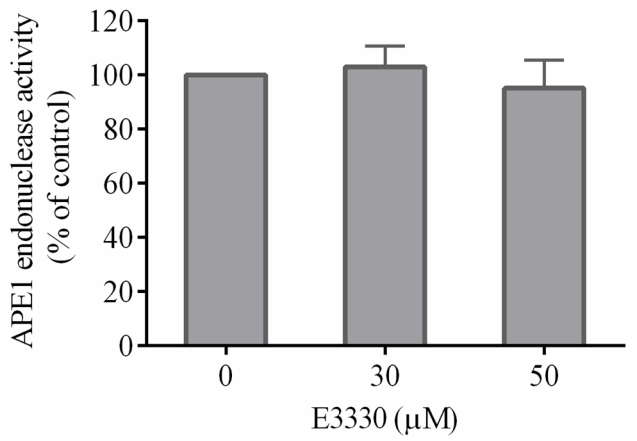


Figure 5.2 APE1 endonuclease activity in the presence of the redox inhibitor E3330. A fluorescence-based enzymatic assay based on the cleavage of a double-stranded DNA containing an abasic site and labeled with a fluorophore (TAMRA) and a quencher (BHQ-2) was performed. Values represent mean ± SD (n = 3) and are expressed as percentage of activity relative to the negative control.

5.4.2. Effect of E3330 alone or in combination with DTX on the viability and colony formation of MDA-MB-231 cells

The cytotoxicity of DTX in MDA-MB-231 cells was first evaluated by the CV staining assay. The cell viability obtained after a 24 h exposure to DTX (0.5–1000 nM) showed a concentration-dependent decrease for concentrations up to 50 nM (Fig. 5.3) and then reached a plateau with higher DTX concentrations. The cytotoxic effect of DTX was significant at concentrations ≥ 5 nM ($P < 0.05$), showing that MDA-MB-231 cells are sensitive to DTX at low nanomolar concentrations. In addition, the MTS reduction assay was used as a confirmatory methodology for the assessment of the cytotoxicity of DTX and the dose-response profiles obtained were similar for both assays (Fig. 5.3).

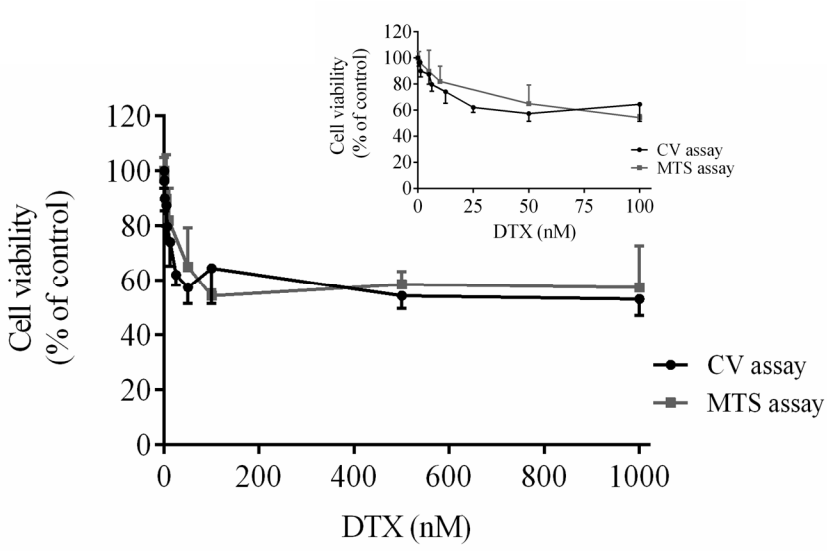


Figure 5.3 Cytotoxic effects of docetaxel (DTX) in MDA-MB-231 cells. The viability of cells treated with DTX for 24 h was evaluated by CV staining (n = 3–7) and MTS reduction (n = 2–3) assays. Inset: effect of concentrations of DTX up to 100 nM. Values represent mean ± SD and are expressed as percentages of vehicle-treated control cells.

The CV and MTS reduction assays revealed that E3330 (30 µM) alone was not cytotoxic for MDA-MB-231 cells (Fig. 5.4A and 5.4B). For the evaluation of the combined treatment of E3330 with DTX, MDA-MB-231 cells were pre-incubated for 2 h with the APE1 redox inhibitor and then simultaneously exposed to DTX at low (0.5 nM) or high (100 nM) clinically achievable concentrations [42–45]. It should be noted that DTX at 0.5 nM induced a decrease in cell viability of only 3.7% in CV assay (N.S.) and 2.6% in the MTS assay (N.S.). E3330 did not modify the viability of DTX-treated cells (Fig. 5.4A and 5.4B).

The colony formation assay was also performed to evaluate the impact of the inhibition of APE1 redox function in the proliferation of cells exposed to DTX. In the conditions tested, the decrease induced by DTX (0.5 nM) in the cell survival of MDA-MB-231 cells was more pronounced than the effects of this chemotherapeutic drug in the cell viability assays (Fig. 5.4). In addition, MDA-MB-231 cells revealed to be highly sensitive to DTX at 10 nM concentration (data not shown). E3330 induced a decrease in the surviving fraction of MDA-MB-231 cells when compared to vehicle-treated control cells (N.S.). Conversely, the combination of E3330 with DTX 0.5 nM revealed a significant decrease in the colony forming ability when compared to vehicle-treated control cells ($P < 0.01$), showing a potential long-term effect of a 24 h exposure to both compounds (Fig. 5.4C).

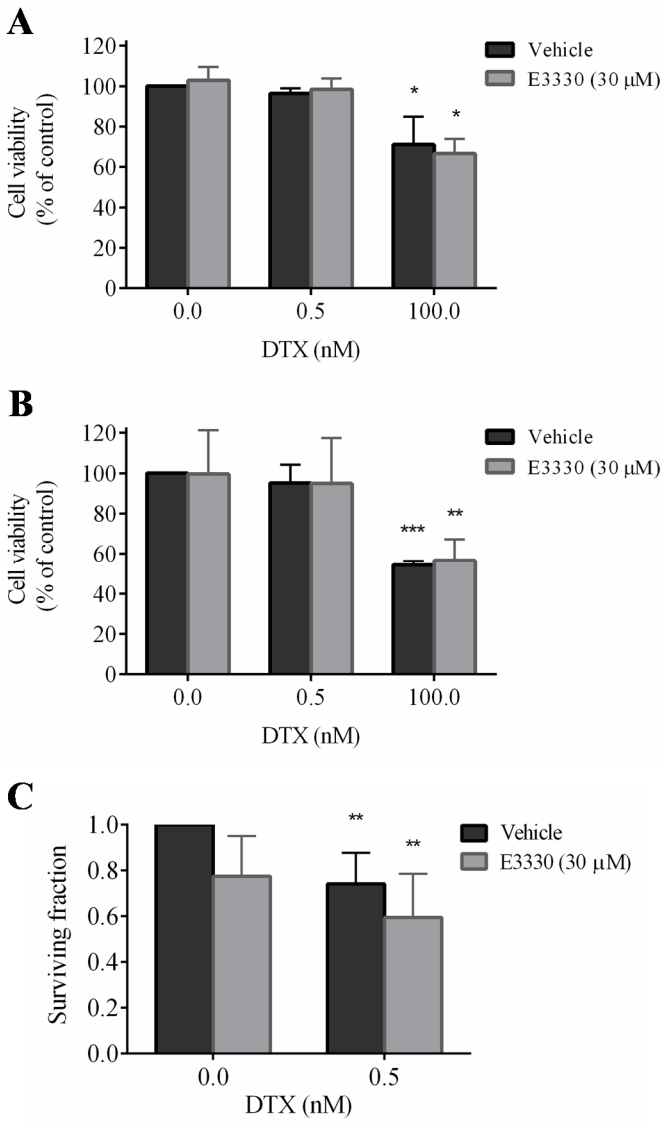


Figure 5.4 Effect of E3330 on viability and colony formation of MDA-MB-231 cells treated with docetaxel (DTX). Cells were pre-incubated with E3330 (30 µM) for 2 h and then simultaneously exposed to E3330 and DTX for 24 h. Cell viability was evaluated by A) CV staining assay and B) MTS reduction assay. Values represent mean ± SD (n = 2–4) and are expressed as percentages relative to vehicle-treated control cells. The colony forming ability was assessed with C) colony formation assay. After the 24 h incubation period with both compounds, MDA-MB-231 cells were grown in drug-free medium for 10–12 days to allow the formation of colonies. Values represent mean ± SD (n = 4). (**P* < 0.05, ***P* < 0.01 and ****P* < 0.001 when compared with vehicle-treated control cells).

5.4.3. Cell cycle distribution of MDA-MB-231 cells treated with E3330 alone or in combination with DTX

The cell DNA content was analysed by flow cytometry to establish the cell cycle distribution of MDA-MB-231 cells exposed to E3330 and/or DTX. The results depicted in Fig. 5.5 show a 2-fold increase in sub-G1 population (*P* < 0.01) and a decrease in G2/M fraction (N.S.) for cells treated with a low DTX concentration (0.5 nM) when compared with vehicle-treated control cells. In addition, DTX at a concentration of 100 nM induced an increase in sub-G1 (7.0 ± 1.0%; *P* < 0.001) and G2/M populations (68.6

± 7.6%; *P* < 0.001) while decreasing G0/G1 (16.8 ± 6.5%; *P* < 0.001) relative to control cells. The APE1 redox inhibitor E3330 (30 µM) did not significantly modify the cell cycle distribution of vehicle-treated or DTX-treated MDA-MB-231 cells. Representative histograms are presented in Fig. 5.5A. All the independent experiments carried out led to coherent results.

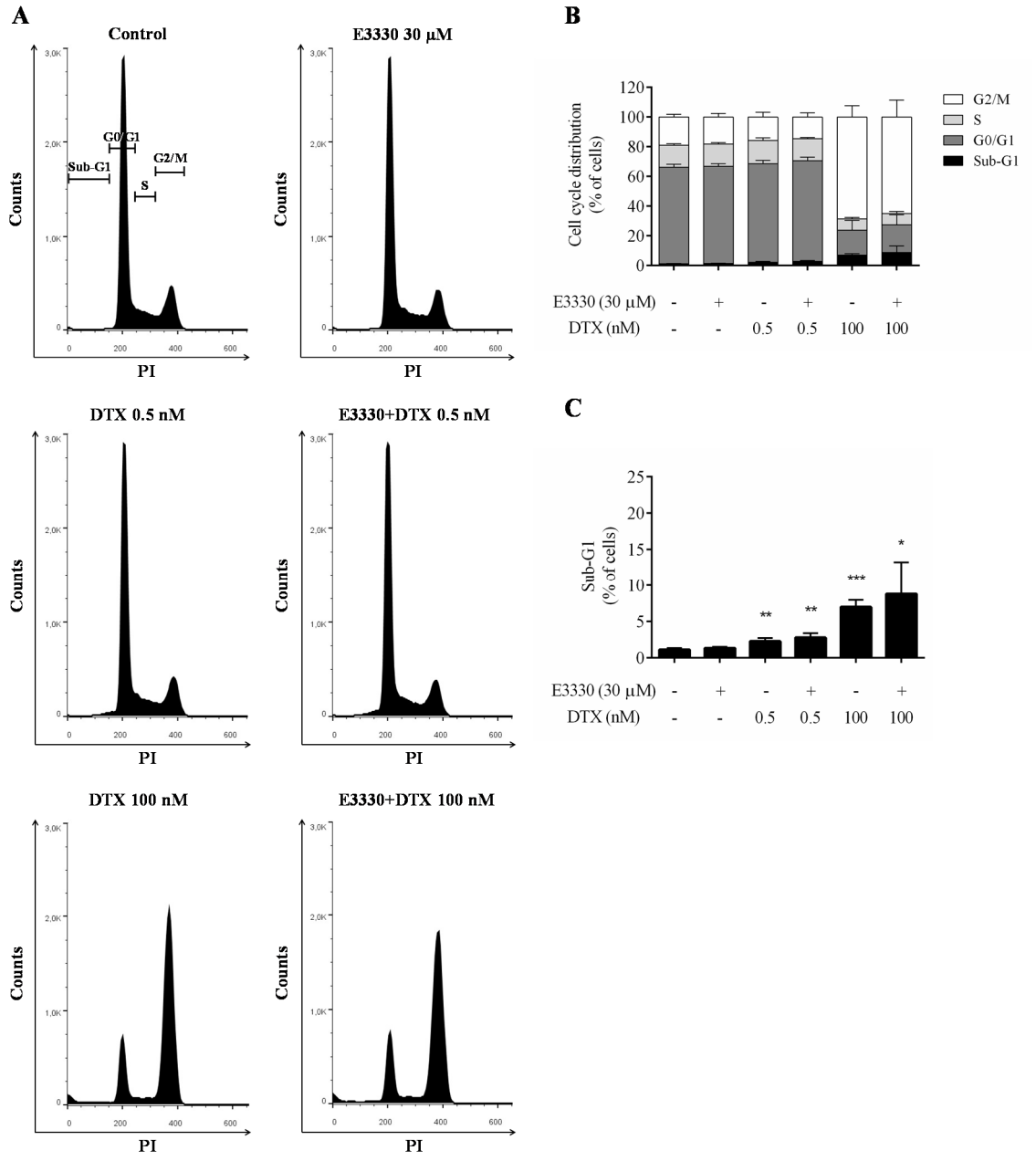


Figure 5.5 Cell cycle distribution of MDA-MB-231 cells treated with E3330 and/ or docetaxel (DTX). Cells were incubated for 2 h with E3330 before the addition of DTX (0.5 or 100 nM). Cell DNA content was analysed by flow cytometry after a 24 h exposure period to both compounds. A) Representative flow cytometry histograms. B) Summary results of sub-G1, G0/G1, S and G2/M populations. C) Sub-G1 population percentage. Values represent mean ± SD (n = 3–4). (**P* < 0.05, ***P* < 0.01 and ****P* < 0.001 when compared with vehicle-treated control cells).

5.4.4. Migration of MDA-MB-231 cells treated with E3330 alone or in combination with DTX

Non-cytotoxic concentrations of E3330 and DTX were selected to evaluate the effect of both compounds on the migration of MDA-MB-231 cells. Collective cell migration was first evaluated by the wound-healing assay. E3330 (30 µM) markedly reduced the % of wound closure to one-third of that of vehicle-treated control cells after a 24 h period of exposure. As shown in Fig. 5.6A, while control cells presented 44.2 ± 9.9% of wound closure, cells treated with the APE1 redox inhibitor displayed 14.6 ± 11.6% of wound closure ($P < 0.001$). DTX (0.5 nM) also decreased cell motility with cultures presenting 33.3 ± 14.4% of wound closure (N.S.). Cells treated with the combination of E3330 and DTX showed a % of wound closure of 17.0 ± 18.3% ($P < 0.05$) which is similar to the decrease in cell motility induced by E3330 alone.

The chemotactic single-cell migration was evaluated by a transwell assay (Fig. 5.6B). E3330 (30 µM) had no effect on the migration of cells exposed to the compound for 24 h. Similarly, the chemotherapeutic drug DTX (0.5 nM) did not significantly modify the migration of vehicle-treated control cells. The simultaneous exposure of cells to E3330 and DTX resulted in 90.9 ± 21.3% of cell migration (N.S.).

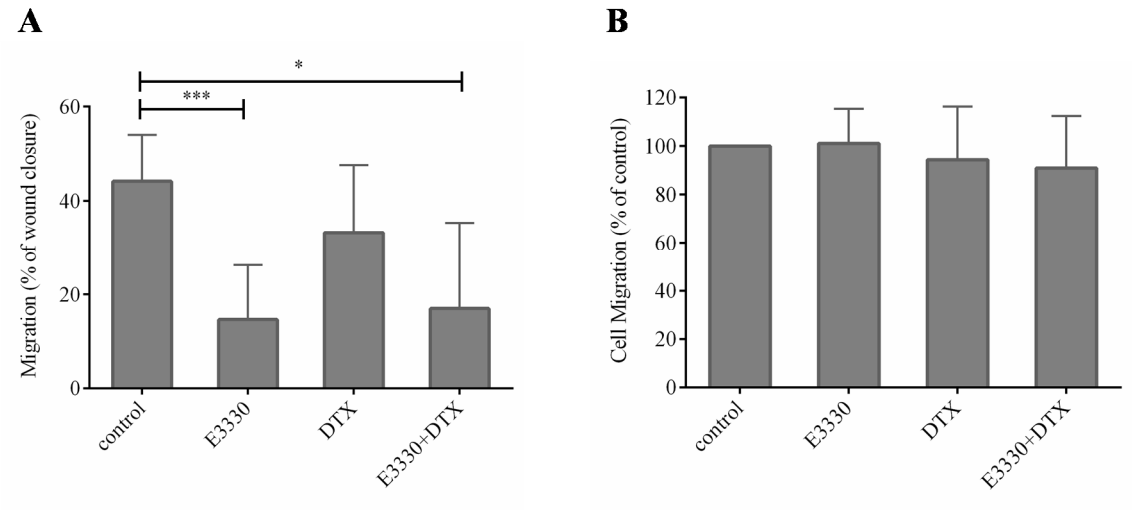


Figure 5.6 Effect of the simultaneous exposure to E3330 (30 µM) and docetaxel (DTX; 0.5 nM) on the migration of MDA-MB-231 cells after a 24 h period of incubation with compounds. Cell migration of MDA-MB-231 cells was evaluated by A) wound healing assay and B) chemotaxis assay. Values represent mean ± SD (n = 4–6). (*$P < 0.05$ and ***$P < 0.001$).

5.4.5. E3330 reduces invasion of DTX-treated MDA-MB-231 cells

The degradation of basement membranes is essential for the invasion of surrounding tissues by cancer cells during the metastatic process. E3330 (30 µM) alone had no

impact on the proteolytic invasion of MDA-MB-231 cells while a relatively non-toxic concentration of DTX (0.5 nM) decreased cell invasion to 85.8 ± 7.3% of vehicle-treated control cells ($P < 0.05$) as shown in Fig. 5.7. However, a more pronounced effect was observed for the combination of E3330 with DTX, which significantly decreased the chemoinvasion to 72.1 ± 3.5% of control cells ($P < 0.001$). When compared with MDA-MB-231 cells exposed only to DTX, the reduction in cell invasion induced by the combined treatment corresponds to a decrease of approximately 13.8% ($P < 0.05$).

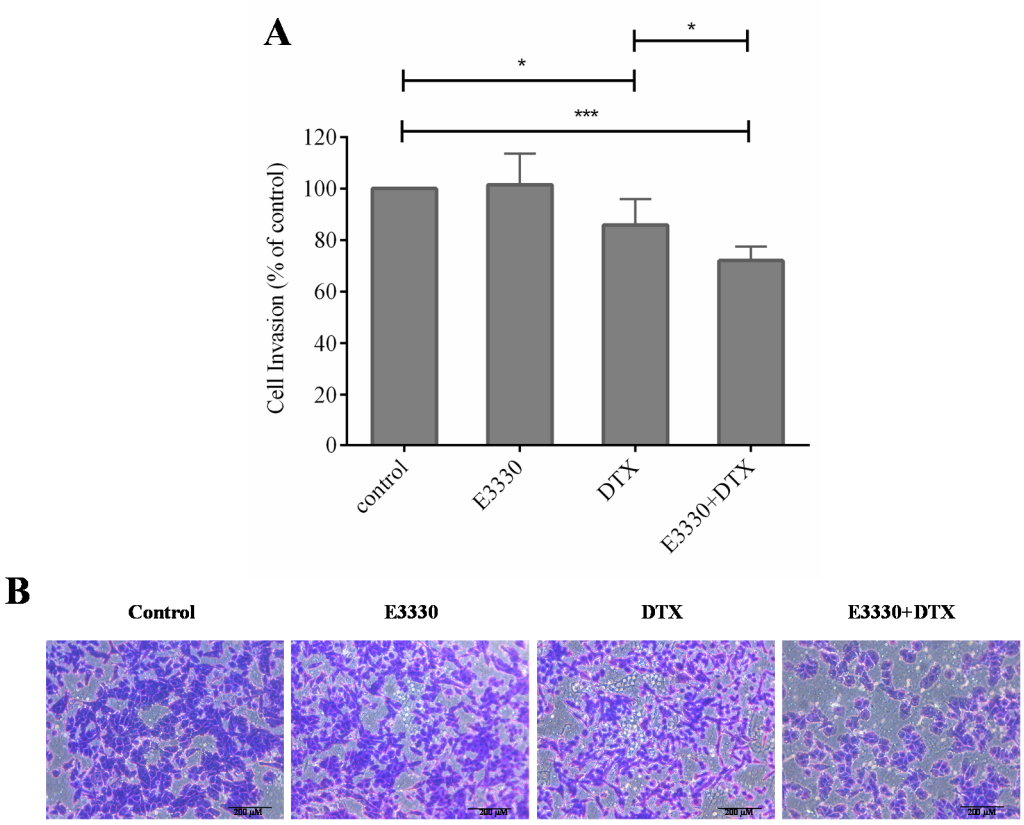


Figure 5.7 Effect of the simultaneous exposure to E3330 (30 µM) and docetaxel (DTX; 0.5 nM) on the invasion of MDA-MB-231 cells. A) Chemoinvasion was evaluated after a 16 h period of incubation with compounds. Values represent mean ± SD (n = 5). B) Representative microscopy images of invading cells stained with crystal violet (scale bar 200 µm). (*$P < 0.05$ and ***$P < 0.001$).

5.5. DISCUSSION

The development of breast cancer metastases involves a sequence of cellular and molecular events, which require the coordinated activation of several signalling pathways. A successful invasion-metastasis cascade initiates with the invasion of surrounding tissues by cells from the primary tumour. In addition, the loss of cell adhesion and the increase of cell motility allow the intravasation into the blood and/or lymphatic vessels, facilitating the dissemination to distant organs. The homing to the metastatic site also requires that surviving circulating tumour cells leave the vessels lumina towards the underlying tissues where they have to survive, regain the ability to proliferate and promote the formation of new vessels (angiogenesis) to generate a secondary tumour (reviewed in [46,47]). The complexity of the metastatic process involves a multiplicity of effector molecules which can be targeted to impair the formation of metastases.

In the last years, APE1 has been studied as a relevant therapeutic target not only because of its role as an endonuclease involved in DNA repair, but also due to its redox function. Although the role of APE1 redox domain is not completely uncovered, several transcription factors and signalling pathways implicated in metastases development have been found to be modulated by APE1's redox function [6,7]. In view of this, the present study aimed to evaluate the role of the combination of DTX with the APE1's redox function inhibitor E3330 on the viability, migration and invasion of metastatic MDA-MB-231 breast cancer cells.

E3330 was the first APE1 redox inhibitor to be identified [25] and it has been the most widely studied. Recently, it was reported that E3330 might bind to the DNA repair active site of APE1 and consequently inhibit the endonuclease activity of the enzyme [32]. However, E3330 seems to be a very weak endonuclease inhibitor since this effect was shown to occur only for concentrations above 100 µM [32,33]. In our study, E3330 at 30 or 50 µM had no effect in APE1 endonuclease activity while these concentrations were reported to inhibit the redox function of APE1 by several authors [14,17,33,48–50]. In addition, in a previous work from our group, E3330 (30 µM) did not modify the viability of MDA-MB-231 cells [20]. Therefore, this concentration was selected to be further used in cell-based assays.

Chemotherapy is the leading treatment for triple-negative advanced breast cancer and taxane-containing regimens are the main therapeutic alternative for metastatic disease refractory to adjuvant anthracycline-based regimens [51].

Taxanes act primarily by binding to the β-subunit of tubulin and inducing the microtubules hyperstabilization which prevents their depolymerization. This disruption of the microtubules dynamics impairs cell proliferation and cell cycle progression and ultimately induces cell death [45,52,53]. In this work, DTX was selected because it has been suggested as the more effective taxane in advanced breast cancer [54]. Moreover, anti-migratory, anti-invasive and anti-angiogenic properties were reported for DTX in different cell lines [55–58].

The dose-response curve of DTX in MDA-MB-231 cells was first established using two different cytotoxicity assays to select the concentrations of the antimicrotubule agent for further assays. The cytotoxic profiles obtained with both CV and MTS assays were similar for the wide range of DTX concentrations tested (0.5–1000 nM). Recently, it was reported a biphasic growth curve for increasing concentrations of DTX in MDA-MB-231 cells with a higher cellular sensitivity at lower concentrations of the drug [45]. Under our experimental conditions MDA-MB-231 cells did not present a biphasic response. In fact, they were sensitive to low nanomolar concentrations but sensitivity to DTX did not increase for concentrations higher than 100 nM, as evidenced by cell viability values above 50%. This concentration-related plateau might be explained because DTX is a cell cycle-specific chemotherapeutic drug and cells were exposed to this compound for 24 h which is a period of time lower than the duration of one cell cycle [59,60]. Consequently, cells are not in the same cell cycle phase and only the proliferating cells are susceptible to the cytotoxic effects of DTX [61]. In this case, an increase of the exposure time should be more effective to decrease cell viability than the increase of drug concentration [62]. In view of this, DTX at 0.5 nM (non-cytotoxic) and/or 100 nM were selected to be tested in combination with E3330. The cell viability was not altered by the addition of E3330 as evaluated by the CV and MTS assays.

Along with the short-term viability assays and since the toxic effects of DTX may be time-dependent, the colony formation assay was performed to evaluate the long-term effects of the combination of E3330 and DTX in the proliferation of breast cancer MDA-MB-231 cells. The colony formation assay revealed a higher sensitivity to DTX when compared to the CV and MTS assays where this level of exposure was non-cytotoxic. A similar result was obtained for E3330 alone which decreased the proliferation of MDA-MB-231 cells in the colony formation assay. Additionally, the combination of the APE1 redox inhibitor with DTX also resulted in a minor decrease in the surviving fraction. These findings highlight the importance of time in the evaluation of drug cytotoxicity as well as the use of multiple endpoints. In addition, our results pointed to a possible sensitising effect of E3330 in DTX-treated cells in terms of colony formation.

Although the molecular mechanisms of DTX action remain to be completely elucidated it is recognized that its cytotoxicity depends on the cell cycle phase as abovementioned. DTX is active in S, G2 and M-phases cells and triggers different forms of cell death depending on drug concentration [45,52,59,60]. Thus, the development of more effective taxane-containing drug combinations for cancer therapy requires the understanding of the impact of each compound in the cell cycle profile. In this context, the effect of E3330 in the cell cycle of MDA-MB-231 cells was evaluated. Our results suggest the absence of alterations in cell cycle progression of MDA-MB-231 cells upon exposure to the APE1 redox inhibitor. Although the modifications induced in cell cycle distribution by small-molecule inhibitors might be cell type-dependent, similar results were reported for E3330 in pancreatic cancer PANC-1 cells [14] and in mitotic arrest deficiency 2 (Mad2) wild-type and deficient-hematopoietic progenitor (HPC) cells [63]. Moreover, Zou *et al.* [14] evaluated the expression of the proliferation antigen Ki-67 in PANC-1 cells and showed that E3330 might induce the exit of cell cycle by promoting the cells entry in G0 phase. More recently, a decrease in the S-phase population and an increase in the levels of p21 protein (cyclin-dependent kinase inhibitor) were described for two pancreatic cancer cell lines (PaCa-2 and PANC-1) after treatment with E3330 suggesting a delay in the progression from G1 to S-phase [29]. These conflicting data emphasise the importance of the future evaluation of the effect of E3330 in the effectors of the signalling pathways controlling the cell cycle to clarify the role of the APE1 redox inhibitor. Regarding the cell cycle profile of DTX in MDA-MB-231 cells, our results presented a minor increase in the sub-G1 population for cells exposed to DTX 0.5 nM. The sub-G1 population corresponds to the accumulation of hypodiploid cells which are usually undergoing cell death mechanisms [45]. In addition to the increase in sub-G1 population, cells treated with DTX 100 nM evidenced the typical G2/M arrest induced by taxanes [45,52,60]. The combined treatment only marginally modified the cell cycle profile of cells exposed to DTX 100 nM.

APE1 is involved in the response to oxidative stress and in the redox regulation of several transcription factors of signalling pathways that may be implicated in tumour progression (reviewed in [6,7]). The levels of APE1 expression were also described to affect the expression of genes involved in cell adhesion, remodeling of extracellular matrix (ECM), reorganization of the actin cytoskeleton, microtubules structure and cell migration [64–66]. In recent studies, the decrease of APE1 expression presented contradictory results in terms of cell migration. The APE1 downregulation was proposed to enhance the migration of human lung carcinoma A549 cells by a

mechanism involving the regulation of TGF-β1 expression [65] while it was reported to reduce the migration and invasion of human hepatocellular carcinoma MHCC97-H cells [67]. Therefore the effects of APE1 in cell motility seem to be cell type-dependent. Nevertheless, the role of APE1 redox activity in cell migration and invasion still has to be clarified.

In the migration assays performed we evaluated non-toxic concentrations of E3330 and/or DTX because an impairment of cell viability would indirectly affect the cell migration. Moreover, E3330 at concentrations ranging from 1 to 30 μM inhibited the migration of pancreatic cancer cells [14] and retinal endothelial cells [30]. The anti-migratory properties of DTX had also been reported to require lower concentrations than those required for the antiproliferative effects [57,68].

A complex set of molecular and morphological modifications have to occur to elicit cell migration [69]. Diverse types of cell migration, involving distinct mechanisms, have been implicated in cancer metastases [70,71]. In the case of breast cancer cells different types of single-cell and of collective cell migration are described [72]. For this reason, two different methodologies, the wound healing and the chemotaxis assays, were adopted to explore the role of E3330 and the combined treatment in the migration of MDA-MB-231 cells. E3330 significantly decreased collective cell migration measured by the wound healing assay but did not affect chemotaxis analysed by the transwell assay. Similarly, DTX slightly reduced the collective cell migration but not chemotaxis. This may be a consequence of the different methodologies and the distinct molecular and cellular mechanisms involved in these types of cell migration. In fact, in the wound healing assay, the horizontal motility of adherent cells is assessed, recapitulating the *in vivo* collective cell migration [69,73], while the chemotaxis assay is focused on the evaluation of the directional cell motility in response to a chemoattractant gradient. The chemotactic migration involves the horizontal and vertical migration and provides information about the single cell motility [69,73]. Moreover, despite the fact that the conditions of the chemotaxis assay better mimic the *in vivo* tumour microenvironment we cannot discard a possible binding of E3330 to the serum proteins [34] contributing to a decrease in the amount of E3330 available to enter in the cells and interact with the enzyme when compared to the wound healing assay.

Along with migration, the ability of cancer cells to carry the proteolytic degradation of the extracellular matrix is essential for the invasion of surrounding tissues and metastases development. The APE1 redox inhibitor alone did not modify the invasion of MDA-MB-231 cells. However when E3330 was combined with DTX a significant

decrease in invasion was observed. Among the numerous players required for cell invasiveness the regulation of the expression of matrix metalloproteinases (MMPs) and the hyaluronan cell-surface receptor CD44 may be responsible for the abovementioned reduction in invasion upon treatment with the compounds. Alterations in the expression of CD44 and MMPs have been correlated with invasive phenotypes of breast cancer [74,75] and they participated in the invasion of MDA-MB-231 cells in *in vitro* models [75,76]. E3330 suppressed the expression of CD44 in pancreatic cancer cells probably *via* inhibition of the AP-1 activation and supporting our hypothesis [7,28]. MMPs expression is regulated by the transcription factor AP-1 and the NF-κB pathway. The last one is particularly relevant for the regulation of MMP-9 [75,77]. The WNT/β-catenin signalling pathway might also modulate the MMPs expression [77]. These effects could be assigned to the blocked AP-1 and NF-κB activation described for the E3330 as well as for the recently attributed role of APE1's redox function in the modulation of WNT/β-catenin signalling pathway [7,18]. Moreover, E3330 is a quinone-based compound which may also induce the generation of ROS [14,18] and APE1 is also involved in the intracellular redox regulation through a Rac-1 regulated NADPH oxidase activation [6,7]. In view of the multiple signalling pathways and cellular functions under ROS control that promote cancer development we cannot exclude a ROS-mediated contribution for the decrease in cell invasion presented by the treatment with E3330 and DTX [7,41,78].

5.6. CONCLUSIONS

In summary, the results presented in this Chapter suggest that E3330 should be further studied as a therapeutic tool in the treatment of advanced breast cancer. This APE1 redox inhibitor reduced cell migration and when used in combination with DTX decreased cell invasion. However, the underlying mechanisms should be further explored by evaluating the alterations in the expression of the effectors from the several signalling pathways that might be affected as a consequence of targeting the APE1 redox function (e.g. NF-κB, AP-1, cadherins, integrins, and MMPs). Furthermore, the complexity of the APE1 interactome network results in the modulation of multiple targets upon inhibition of APE1 redox function that can have differential effects according to the cell type. Their knowledge is essential for the prediction of potential side effects and for the translational development of E3330 as an anticancer agent.

5.7. REFERENCES

1. DeSantis C, Bray F, Ferlay J. International variation in female breast cancer incidence and mortality rates. Cancer Epidemiol Biomarkers Prev. 2015;24:1495–1506.

2. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer. 2015;136:E359–E386.

3. Weigelt B, Peterse JL, van 't Veer LJ. Breast cancer metastasis: markers and models. Nat Rev Cancer. 2005;5:591–602.

4. Eccles SA, Aboagye EO, Ali S, Anderson AS, Armes J, Berditchevski F, et al. Critical research gaps and translational priorities for the successful prevention and treatment of breast cancer. Breast Cancer Res. 2013;15:R92.

5. Scully OJ, Bay B-H, Yip G, Yu Y. Breast Cancer Metastasis. Cancer Genomics Proteomics. 2012;9:311–320.

6. Tell G, Fantini D, Quadrifoglio F. Understanding different functions of mammalian AP endonuclease (APE1) as a promising tool for cancer treatment. Cell Mol Life Sci. 2010;67:3589–3608.

7. Kelley MR, Georgiadis MM, Fishel ML. APE1/Ref-1 role in redox signaling: translational applications of targeting the redox function of the DNA repair/redox protein APE1/Ref-1. Curr Mol Pharmacol. 2012;5:36–53.

8. Nishi T, Shimizu N, Hiramoto M, Sato I, Yamaguchi Y, Hasegawa M, et al. Spatial redox regulation of a critical cysteine residue of NF-kappa B in vivo. J Biol Chem. 2002;277:44548–44556.

9. Xanthoudakis S, Curran T. Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity. EMBO J. 1992;11:653–665.

10. Huang RP, Adamson ED. Characterization of the DNA-binding properties of the early growth response-1 (Egr-1) transcription factor: evidence for modulation by a redox mechanism. DNA Cell Biol. 1993;12:265–273.

11. Pines A, Bivi N, Romanello M, Damante G, Kelley MR, Adamson ED, et al. Cross-regulation between Egr-1 and APE/Ref-1 during early response to oxidative stress in the human osteoblastic HOBIT cell line: evidence for an autoregulatory loop. Free Radic Res. 2005;39:269–281.

12. Huang LE, Arany Z, Livingston DM, Bunn HF. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. J Biol Chem. 1996;271:32253–32259.

13. Gray MJ, Zhang J, Ellis LM, Semenza GL, Evans DB, Watowich SS, et al. HIF-1alpha, STAT3, CBP/p300 and Ref-1/APE are components of a transcriptional complex that regulates Src-dependent hypoxia-induced expression of VEGF in pancreatic and prostate carcinomas. Oncogene. 2005;24:3110–3120.

14. Zou GM, Maitra A. Small-molecule inhibitor of the AP endonuclease 1/REF-1 E3330 inhibits pancreatic cancer cell growth and migration. Mol Cancer Ther. 2008;7:2012–2021.

15. Jayaraman L, Murthy KG, Zhu C, Curran T, Xanthoudakis S, Prives C. Identification of redox/repair protein Ref-1 as a potent activator of p53. Genes Dev. 1997;11:558–570.

16. Gaiddon C, Moorthy NC, Prives C. Ref-1 regulates the transactivation and pro-apoptotic functions of p53 in vivo. EMBO J. 1999;18:5609–5621.

17. Cardoso AA, Jiang Y, Luo M, Reed AM, Shahda S, He Y, et al. APE1/Ref-1 regulates STAT3 transcriptional activity and APE1/Ref-1-STAT3 dual-targeting effectively inhibits pancreatic cancer cell survival. PLoS One. 2012;7:e47462.

18. Jiang S, Zhu L, Tang H, Zhang M, Chen Z, Fei J, et al. Ape1 regulates WNT/β-catenin signaling through its redox functional domain in pancreatic cancer cells. Int J Oncol. 2015;47:610–620.

19. Abbotts R, Madhusudan S. Human AP endonuclease 1 (APE1): from mechanistic insights to druggable target in cancer. Cancer Treat Rev. 2010;36:425–435.

20. Guerreiro PS, Fernandes AS, Costa JG, Castro M, Miranda JP, Oliveira NG. Differential effects of methoxyamine on doxorubicin cytotoxicity and genotoxicity in MDA-MB-231 human breast cancer cells. Mutat Res. 2013;757:140–147.

21. Kakolyris S, Kaklamanis L, Engels K, Fox SB, Taylor M, Hickson ID, et al. Human AP endonuclease 1 (HAP1) protein expression in breast cancer correlates with lymph node status and angiogenesis. Br J Cancer. 1998;77:1169–1173.

22. Puglisi F, Aprile G, Minisini AM, Barbone F, Cataldi P, Tell G, et al. Prognostic significance of Ape1/ref-1 subcellular localization in non-small cell lung carcinomas. Anticancer Res. 2001;21:4041–4049.

23. Coskun E, Jaruga P, Reddy PT, Dizdaroglu M. Extreme expression of DNA repair protein apurinic/apyrimidinic endonuclease 1 (APE1) in human breast cancer as measured by liquid chromatography and isotope dilution tandem mass spectrometry. Biochemistry. 2015;54:5787–5790.

24. Rodrigues AS, Gomes BC, Martins C, Gromicho M, Oliveira NG, Guerreiro PS, et al. DNA repair and resistance to cancer therapy. In: Chen C, editor. New Research Directions in DNA Repair. InTech; 2013. pp.489–528.

25. Shimizu N, Sugimoto K, Tang J, Nishi T, Sato I, Hiramoto M, et al. High-performance affinity beads for identifying drug receptors. Nat Biotechnol. 2000;18:877–881.

26. Jedinak A, Dudhgaonkar S, Kelley MR, Sliva D. Apurinic/Apyrimidinic endonuclease 1 regulates inflammatory response in macrophages. Anticancer Res. 2011;31:379–385.

27. Fishel ML, Colvin ES, Luo M, Kelley MR, Robertson KA. Inhibition of the redox function of APE1/Ref-1 in myeloid leukemia cell lines results in a hypersensitive response to retinoic acid-induced differentiation and apoptosis. Exp Hematol. 2010;38:1178–1188.

28. Zou GM, Karikari C, Kabe Y, Handa H, Anders RA, Maitra A. The Ape-1/Ref-1 redox antagonist E3330 inhibits the growth of tumor endothelium and endothelial progenitor cells: therapeutic implications in tumor angiogenesis. J Cell Physiol. 2009;219:209–218.

29. Fishel ML, Jiang Y, Rajeshkumar NV, Scandura G, Sinn AL, He Y, et al. Impact of APE1/Ref-1 redox inhibition on pancreatic tumor growth. Mol Cancer Ther. 2011;10:1698–1708.

30. Jiang A, Gao H, Kelley MR, Qiao X. Inhibition of APE1/Ref-1 redox activity with APX3330 blocks retinal angiogenesis in vitro and in vivo. Vision Res. 2011;51:93–100.

31. Su D, Delaplane S, Luo M, Rempel DL, Vu B, Kelley MR, et al. Interactions of apurinic/apyrimidinic endonuclease with a redox inhibitor: evidence for an alternate conformation of the enzyme. Biochemistry. 2011;50:82–92.

32. Manvilla BA, Wauchope O, Seley-Radtke KL, Drohat AC. NMR studies reveal an unexpected binding site for a redox inhibitor of AP endonuclease 1. Biochemistry. 2011;50:10540–10549.

33. Zhang J, Luo M, Marasco D, Logsdon D, LaFavers KA, Chen Q, et al. Inhibition of apurinic/apyrimidinic endonuclease I's redox activity revisited. Biochemistry. 2013;52:2955–2966.

34. Cesaratto L, Codarin E, Vascotto C, Leonardi A, Kelley MR, Tiribelli C, et al. Specific inhibition of the redox activity of ape1/ref-1 by E3330 blocks TNF-α-induced activation of IL-8 production in liver cancer cell lines. PLoS One. 2013;8:e70909.

35. Li Y, Liu X, Zhou T, Kelley MR, Edwards PA, Gao H, et al. Suppression of choroidal neovascularization through inhibition of APE1/Ref-1 redox activity. Invest Ophthalmol Vis Sci. 2014;55:4461–4469.

36. Li Y, Liu X, Zhou T, Kelley MR, Edwards P, Gao H, et al. Inhibition of APE1/Ref-1 redox activity rescues human retinal pigment epithelial cells from oxidative stress and reduces choroidal neovascularization. Redox Biol. 2014;2:485–494.

37. Simeonov A, Kulkarni A, Dorjsuren D, Jadhav A, Shen M, McNeill DR, et al. Identification and characterization of inhibitors of human apurinic/apyrimidinic endonuclease APE1. PLoS One. 2009;4:e5740.

38. Fernandes AS, Gaspar J, Cabral MF, Rueff J, Castro M, Batinic-Haberle I, et al. Protective role of ortho-substituted Mn(III) N-alkylpyridylporphyrins against the oxidative injury induced by tert-butylhydroperoxide. Free Radic Res. 2010;44:430–440.

39. Fernandes AS, Serejo J, Gaspar J, Cabral F, Bettencourt AF, Rueff J, et al. Oxidative injury in V79 Chinese hamster cells: protective role of the superoxide dismutase mimetic MnTM-4-PyP. Cell Biol Toxicol. 2010;26:91–101.

40. Costa JG, Saraiva N, Guerreiro PS, Louro H, Silva MJ, Miranda JP, et al. Ochratoxin A-induced cytotoxicity, genotoxicity and reactive oxygen species in kidney cells: An integrative approach of complementary endpoints. Food Chem Toxicol. 2016;87:65–76.

41. Fernandes AS, Flórido A, Saraiva N, Cerqueira S, Ramalhete S, Cipriano M, et al. Role of the copper(II) complex Cu[15]pyN5 in intracellular ROS and breast cancer cell motility and invasion. Chem Biol Drug Des. 2015;86:578–588.

42. Baker SD, Zhao M, Lee CKK, Verweij J, Zabelina Y, Brahmer JR, et al. Comparative pharmacokinetics of weekly and every-three-weeks docetaxel. Clin Cancer Res. 2004;10:1976–1983.

43. Andersen A, Warren DJ, Brunsvig PF, Aamdal S, Kristensen GB, Olsen H. High sensitivity assays for docetaxel and paclitaxel in plasma using solid-phase extraction and high-performance liquid chromatography with UV detection. BMC Clin Pharmacol. 2006;6:2.

44. Brunsvig PFR, Andersen A, Aamdal S, Kristensen V, Olsen H. Pharmacokinetic analysis of two different docetaxel dose levels in patients with non-small cell lung cancer treated with docetaxel as monotherapy or with concurrent radiotherapy. BMC Cancer. 2007;7:197.

45. Hernández-Vargas H, Palacios J, Moreno-Bueno G. Molecular profiling of docetaxel cytotoxicity in breast cancer cells: uncoupling of aberrant mitosis and apoptosis. Oncogene. 2007;26:2902–2913.

46. Gupta GP, Massagué J. Cancer metastasis: building a framework. Cell. 2006;127:679–695.

47. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. Cell. 2011;147:275–292.

48. Luo M, Delaplane S, Jiang A, Reed A, He Y, Fishel M, et al. Role of the multifunctional DNA repair and redox signaling protein Ape1/Ref-1 in cancer and endothelial cells: small-molecule inhibition of the redox function of Ape1. Antioxid Redox Signal. 2008;10:1853–67.

49. Luo M, Zhang J, He H, Su D, Chen Q, Gross ML, et al. Characterization of the redox activity and disulfide bond formation in apurinic/apyrimidinic endonuclease. Biochemistry. 2012;51:695–705.

50. Kelley MR, Luo M, Reed A, Su D, Delaplane S, Borch RF, et al. Functional analysis of novel analogues of E3330 that block the redox signaling activity of the multifunctional AP endonuclease/redox signaling enzyme APE1/Ref-1. Antioxid Redox Signal. 2011;14:1387–1401.

51. Cardoso F, Harbeck N, Fallowfield L, Kyriakides S, Senkus E, ESMO Guidelines Working Group. Locally recurrent or metastatic breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2012;23 Suppl 7:vii11–vii19.

52. McGrogan BT, Gilmartin B, Carney DN, McCann A. Taxanes, microtubules and chemoresistant breast cancer. Biochim Biophys Acta. 2008;1785:96–132.

53. Herbst RS, Khuri FR. Mode of action of docetaxel - a basis for combination with novel anticancer agents. Cancer Treat Rev. 2003;29:407–415.

54. Crown J, O'Leary M, Ooi W-S. Docetaxel and paclitaxel in the treatment of breast cancer: a review of clinical experience. Oncologist. 2004;9 Suppl 2:24–32.

55. Benelli R, Monteghirfo S, Balbi C, Barboro P, Ferrari N. Novel antivascular efficacy of metronomic docetaxel therapy in prostate cancer: hnRNP K as a player. Int J Cancer. 2009;124:2989–2996.

56. Kogashiwa Y, Sakurai H, Kimura T, Kohno N. Docetaxel suppresses invasiveness of head and neck cancer cells in vitro. Cancer Sci. 2010;101:1382–1386.

57. Kamath K, Smiyun G, Wilson L, Jordan MA. Mechanisms of inhibition of endothelial cell migration by taxanes. Cytoskeleton (Hoboken). 2014;71:46–60.

58. Ahmad A, Ali S, Wang Z, Ali AS, Sethi S, Sakr WA, et al. 3,3'-Diindolylmethane enhances taxotere-induced growth inhibition of breast cancer cells through downregulation of FoxM1. Int J Cancer. 2011;129:1781–1791.

59. Hennequin C, Giocanti N, Favaudon V. S-phase specificity of cell killing by docetaxel (Taxotere) in synchronised HeLa cells. Br J Cancer. 1995;71:1194–1198.

60. Gligorov J, Lotz JP. Preclinical pharmacology of the taxanes: implications of the differences. Oncologist. 2004;9 Suppl 2:3–8.

61. Shah MA, Schwartz GK. Cell cycle-mediated drug resistance: an emerging concept in cancer therapy. Clin Cancer Res. 2001;7:2168–2181.

62. Hill BT, Whelan RD, Shellard SA, McClean S, Hosking LK. Differential cytotoxic effects of docetaxel in a range of mammalian tumor cell lines and certain drug resistant sublines in vitro. Invest New Drugs. 1994;12:169–182.

63. Rohrabaugh SL, Hangoc G, Kelley MR, Broxmeyer HE. Mad2 haploinsufficiency protects hematopoietic progenitor cells subjected to cell-cycle stress in vivo and to inhibition of redox function of Ape1/Ref-1 in vitro. Exp Hematol. 2011;39:415–423.

64. Vascotto C, Cesaratto L, Zeef LAH, Deganuto M, D'Ambrosio C, Scaloni A, et al. Genome-wide analysis and proteomic studies reveal APE1/Ref-1 multifunctional role in mammalian cells. Proteomics. 2009;9:1058–1074.

65. Sakai Y, Yamamori T, Yasui H, Inanami O. Downregulation of the DNA repair enzyme apurinic/apyrimidinic endonuclease 1 stimulates transforming growth factor-β1 production and promotes actin rearrangement. Biochem Biophys Res Commun. 2015;461:35–41.

66. Yamauchi A, Kawabe J, Kabara M, Matsuki M, Asanome A, Aonuma T, et al. Apurinic/apyrimidinic endonucelase 1 maintains adhesion of endothelial progenitor cells and reduces neointima formation. Am J Physiol Heart Circ Physiol. 2013;305:H1158–H1167.

67. Zheng Z-H, Du W, Li Y-J, Gao M-Q, Huang A-M, Liu J-F. Lentiviral-mediated short hairpin RNA silencing of APE1 suppresses hepatocellular carcinoma proliferation and migration: A potential therapeutic target for hepatoma treatment. Oncol Rep. 2015;34:95–102.

68. Hotchkiss KA, Ashton AW, Mahmood R, Russell RG, Sparano JA, Schwartz EL. Inhibition of endothelial cell function in vitro and angiogenesis in vivo by docetaxel (Taxotere): association with impaired repositioning of the microtubule organizing center. Mol Cancer Ther. 2002;1:1191–1200.

69. Friedl P, Alexander S. Cancer invasion and the microenvironment: plasticity and reciprocity. Cell. 2011;147:992–1009.

70. Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. Nat Rev Cancer. 2003;3:362–374.

71. Yilmaz M, Christofori G. Mechanisms of motility in metastasizing cells. Mol Cancer Res. 2010;8:629–642.

72. Roussos ET, Condeelis JS, Patsialou A. Chemotaxis in cancer. Nat Rev Cancer. 2011;11:573–587.

73. Kramer N, Walzl A, Unger C, Rosner M, Krupitza G, Hengstschläger M, et al. In vitro cell migration and invasion assays. Mutat Res. 752: 10–24.

74. Götte M, Yip GW. Heparanase, hyaluronan, and CD44 in cancers: a breast carcinoma perspective. Cancer Res. 2006;66:10233–10237.

75. Balduyck M, Zerimech F, Gouyer V, Lemaire R, Hemon B, Grard G, et al. Specific expression of matrix metalloproteinases 1, 3, 9 and 13 associated with invasiveness of breast cancer cells in vitro. Clin Exp Metastasis. 2000;18:171–178.

76. Montgomery N, Hill A, McFarlane S, Neisen J, O'Grady A, Conlon S, et al. CD44 enhances invasion of basal-like breast cancer cells by upregulating serine protease and collagen-degrading enzymatic expression and activity. Breast Cancer Res. 2012;14:R84.

77. Yan C, Boyd DD. Regulation of matrix metalloproteinase gene expression. J Cell Physiol. 2007;211:19–26.

78. Acharya A, Das I, Chandhok D, Saha T. Redox regulation in cancer: a double-edged sword with therapeutic potential. Oxid Med Cell Longev. 2010;3:23–34.

CHAPTER 6

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

APE1 is undoubtedly an enzyme of interest in different human diseases particularly in cancer field. During the last decades, APE1 has been regarded as the major endonuclease with a fundamental role in DNA repair. Targeting APE1 is fully justified by an increase in toxic lesions such as AP sites, which culminate in subsequent DNA strand breaks if left unrepaired. These lesions can thus contribute to boost the cytotoxic effects and cell death triggered by standard anticancer agents. Moreover, the APE1 endonuclease activity inhibition by small molecules may constitute the rationale of different synthetic lethality strategies that aim to exploit DNA repair deficiencies in a given pathway along with the use of specific DNA repair inhibitors. Therefore, being the multifunctional enzyme APE1 an important target in the emerging field of DNA repair and a relevant therapeutic approach in cancer, we herein explored the *in vitro* modulation of APE1 for breast cancer therapy. Since the importance of APE1 in cancer is currently considered beyond its function in DNA repair, in this work the additional major role of APE1 in the redox modulation of key events in cancer progression and dissemination, namely cell migration and invasion was also addressed in the context of breast cancer.

Breast cancer is still one of the most frequent malignancies, ranking as the fifth cause of cancer-related deaths in the world [1–3]. This disease is the most common cancer in women with the metastases development being the major cause of its high mortality rate. Although the improvements in treatment and diagnosis strategies, a remarkable number of breast tumours are already metastatic at diagnosis, being the majority of these cancers incurable [2,3].

Diverse findings have linked APE1 to breast cancer including the aforementioned differential expression levels of APE1 in malignant and non-tumoural tissues and the dysregulation of the intracellular distribution of this BER enzyme. In fact, the alterations in the subcellular localization of APE1 in tumours relatively to the normal tissues have been associated to higher aggressiveness and poor differentiation in breast cancers hampering the prognosis [4–6].

Human cancer cell lines have been widely used for study genetics and cancer biology, biomarkers development and novel therapies in tumours of different origins. MDA-MB-231 cell line is a well-established and characterized human breast cancer line being recognized as a widely accepted metastatic cancer model as displayed by the large number of studies published in the last decades. It is considered a representative model of poorly differentiated and highly aggressive triple-negative breast cancer (TNBC) [7,8]. In addition to lacking the expression of progesterone receptor, estrogen

receptor and the amplification of the human epidermal growth factor receptor 2 (HER2/neu) typical of TNBC, MDA-MB-231 cells present a mutated p53 gene [7]. These cells are also highly invasive in vitro and tumorigenic in vivo [9–12], being one of the most commonly studied cell line particularly useful to the identification of cancer-related genes, tumour markers of low and high aggressiveness and also in the drug discovery field [13,14]. In fact, these cells have been used in studies focused on the development of DNA repair inhibitors (e.g. PARP inhibitors) and compounds designed to abrogate the invasion and metastases progression [15–19]. In addition, in this work the human breast epithelial MCF10A cell line was also included as a non-tumourigenic cell type. These cells were specifically used to assess the cytotoxicity of the novel small-molecule inhibitors of APE1 endonuclease activity (Chapter 4). Over the years, these cells have been the most used *in vitro* model of normal-like breast cells. MCF10A cells are notably useful to investigate the normal human breast cell function and malignant transformation. They have also been particularly relevant in the Toxicology field, to explore the toxicological effects of xenobiotics with tropism for breast tissue, including some reports from our research group that employed these cells to study current toxicological issues and to obtain mechanistic insights on glycidamide [20] and cadmium deleterious effects [21].

The treatment of triple-negative advanced breast cancer is almost exclusively based in DNA-damaging chemotherapeutic drugs. Currently, in the vast majority of metastatic TNBC tumours, the primary goal of therapy is palliative to improve the quality of life and extend the patient survival. In this type of cancer, anthracyclines as single agents or in combination regimens as well as taxanes are the mainstay treatment for advanced breast cancer [2,3].

Dox is the most commonly used anthracycline antibiotic in numerous types of haematological malignancies and solid tumours, including breast cancer. Although being considered one of the most potent approved anticancer agents, tumour resistance to treatment and the toxicity profile are often unfavourable with the development of life-threatening cardiotoxicity [22]. Among the multiple and complex mechanisms currently proposed for Dox antitumour effects [23,24], the anthracycline-mediated ROS generation may produce oxidised DNA bases that similarly to Dox-induced strand breaks can be repaired by BER pathway. Moreover, Dox has also well-established clastogenic and aneugenic properties being a recognized genotoxic agent. Overall, these features render Dox as a suitable candidate to be evaluated in combination with APE1 inhibitors in a strategy to improve the outcomes of Dox therapy.

On the other hand, taxane-based regimens are the main therapeutic alternative for patients with refractory disease to anthracycline-based regimens [2,3]. Taxanes are microtubule-targeting drugs since their primary mechanism of action is based on tubulin binding resulting in microtubules hyperstabilisation and disrupting their dynamic. Although using a completely different mechanism in comparison with Dox, taxanes also induce cell death by apoptotic and non-apoptotic processes [25]. Some evidences suggested that taxanes can also increase the intracellular ROS which can be involved in the impairment of cell proliferation, migration, invasion and angiogenesis attributed to these anticancer drugs [26,27]. Among the different drugs of this chemotherapeutic class DTX has been highlighted as the more effective compound in advanced breast cancer, being more potent than paclitaxel, the first isolated taxane [28], which is also widely used in cancer therapy.

Despite the different functions of APE1, modulation of its endonuclease activity has been the most exploited towards a novel strategy in cancer treatment. In the presence of the numerous evidences described in Chapter 1 emphasising the potential role of the inhibition of APE1 endonuclease activity to sensitise different types of cancer cells to the standard anticancer agents, this work was firstly delineated to evaluate the effects of commercially available APE1 inhibitors in the cytotoxicity and genotoxicity of Dox (Chapter 3) and to identify novel APE1 endonuclease inhibitors using an *in sillico* approach (Chapter 4).

MX is a commercially available indirect inhibitor of APE1 endonuclease activity widely evaluated in the preclinical setting and the only reported small-molecule inhibitor of APE1 DNA repair function that has been studied in phase I clinical trials. Therefore, this compound was selected to be used in combination with Dox. It should be noted that the therapeutic value of APE1 modulation in combination with Dox has not yet been fully investigated.

In Chapter 3, distinct exposure schedules were established and different concentrations were evaluated to resemble the drug administration and levels achievable in the clinical setting. Cell viability was assessed with two mechanistically different methods. Long-term effects in proliferation of MDA-MB-231 cells were evaluated with the colony formation assay which has been widely regarded as the “gold standard” method in radiobiology and to study chemotherapeutic drugs in tumour cells. This method unveils cells that maintained the dividing and proliferation abilities to yield a large colony of progeny cells after a treatment expected to induce reproductive cell death [29]. Therefore, colony formation assay is able to distinguish cells retaining some

capacity to synthesise biomolecules, such as proteins and DNA but unable to indefinitely divide which could be considered viable in cell viability assays based on enzymatic reactions catalysed by living cells as occurs in the MTT assay. Since the aforementioned compounds are DNA-damaging agents, the genotoxicity assessment is also of utmost importance. The CBMN assay was used to evaluate the effects of APE1 endonuclease inhibitor in combination with anticancer drugs at the chromosome damage level. This emerging assay is the most useful method to determine the micronucleus frequency and it has been widely used in genetic toxicology to identify both clastogenic and aneugenic compounds [30]. Moreover, the CBMN assay is also able to provide insights about the cytostatic effects of compounds [30].

Although MX was not able to clearly sensitise MDA-MB-231 cells to Dox in terms of cell viability and proliferation, having only little effects, this APE1 inhibitor significantly increased the chromosomal damage by promoting the accumulation of unrepaired lesions upon Dox treatment. The apparent ability of MDA-MB-231 cells to tolerate additional genotoxic Dox-inflicted lesions in the presence of MX may preclude the use of this strategy in the clinical setting. The multiple mechanisms of action of Dox which can have different contributions for its cytotoxic and genotoxic properties may contribute for these differential results. In fact, different types of DNA damage have been associated to Dox with the involvement of different DNA repair pathways to cope with the Dox-induced lesions (Chapter 1). Moreover, the recognized redundancy of DNA repair pathways may have a role in the absence of more pronounced effects for the combination of MX with Dox. Additionally, the effects of known anticancer agents and DNA repair inhibitors may be cell-type specific due to the intrinsic DNA repair status of different tumour cells which is translated in distinct responses to these compounds. The importance of these aspects has been highlighted for the stumbling of some DNA repair inhibitors in clinical trials although promising results obtained in their preclinical development [31,32]. Furthermore, APE1 is the major endonuclease in human cells but a second endonuclease APE2 was also identified [33]. Conversely to APE1, APE2 has a weak endonuclease activity but displays a strong 3'-exonuclease and 3'-phosphodiesterase activity [34]. Despite being a minor endonuclease and its cellular roles are unclear, APE2 has been shown to interact with PCNA in the repair of oxidatively damaged DNA (e.g. 8-oxoG) [35]. APE2 also seems to be required for the recovery of lymphoid progenitors following bone marrow depletion by 5-FU [36]. Consequently, a possible contribution of APE2 to the repair of Dox-induced DNA damage via ROS generation should not be neglected. In view of this, to further elucidate the mechanisms underlying the differential effects of MX on Dox cytotoxicity

and genotoxicity, additional studies should be performed, addressing this issue by using other cell types and regarding gene expression of cell death and DNA repair pathways.

Since direct enzyme inhibitors are considered better drug candidates due to their higher specificity with a subsequent decrease in potential side-effects we also attempted to evaluate the putative effects of CRT0044876, a direct inhibitor of APE1 endonuclease activity, in combination with Dox or H_2O_2. CRT0044876 was the first direct inhibitor of APE1 DNA repair function to be identified and described [37]. Nevertheless, this compound did not decrease the viability of cells treated with our oxidative DNA lesions inducer model H_2O_2 or Dox (data not shown). Moreover, the role of CRT0044876 as an APE1 inhibitor is not completely elucidated and some authors were not able to reproduce the *in vitro* results previously reported for this compound [38,39].

Besides CRT0044876 several other compounds have been identified as direct inhibitors of APE1 over the last years. However, evidences of their effectiveness as APE1 inhibitors are also still lacking, precluding their clinical application. In this context, the work herein presented also aimed at the identification of novel small-molecule inhibitors targeting directly APE1 endonuclease activity (Chapter 4).

Computational tools have an overriding role in early stages of drug discovery and development. Since APE1 crystallographic structures were already resolved and essential amino acids of the active site had also been identified, a SBVS protocol based on molecular docking studies was established in this work to identify novel small-molecule inhibitors of APE1 endonuclease activity.

SBVS adopts different modelling techniques to exploit the binding interactions and consequently the molecular recognition between ligands and the target protein. A SBVS approach was preferred over a ligand-based strategy which is based on similarities of known ligands due to the large chemical diversity of compounds described as APE1 inhibitors by other authors. The selection of the most suitable central similarity-property for optimisation of potential ligands to the identification of novel bioactive molecules was thus hampered. Although a higher resolution APE1 X-ray structure was available by the time of the beginning of this work, the APE1 crystallographic structure selected for the docking analysis also displays a high resolution, and importantly it has no missing residues in the catalytic domain being also obtained at a physiological pH. More recently, a higher resolution APE1 structure solved at neutral pH was published [40]. Since this work was already initiated by the

time of the novel APE1 structure discovery, to validate the selection of the aforementioned structure and to ensure that important information was not missing, the position of catalytic active site ion was compared in both structures which showed an almost complete overlapping.

The SBVS and docking studies were performed with compounds from the NCI repository. More than fifty compounds were selected to be acquired after docking analysis followed by visual inspection regarding appropriate binding conformations, fitting into the active site pocket and relevant interactions with key enzyme residues. Poor solubility and precipitation issues in assay conditions precluded the evaluation of all acquired compounds. From the thirty compounds tested in the fluorescence-based APE1 endonuclease activity assay, three compounds, namely compound 22, 37 and 41 inhibited human recombinant APE1 and displayed high purity as evaluated by HPLC-DAD. Compound 22 presented a remarkable inhibition of APE1 activity in the nanomolar range with an IC_{50} value of about 250 nM while compounds 37 and 41 were active at micromolar concentrations. These promising results were recapitulated with a methodological distinct counterscreen assay, thus confirming their potential as APE1 inhibitors.

Compound 22 is among the most potent inhibitors of APE1 identified to date [37,41–43,38,44–50]. The higher potency of compound 22 may be associated to the simultaneous presence of a biphenyl core with sulfonate moieties and an isothiocyanate group producing a more suitable compound positioning into APE1 active site. Several evidences in *in vitro* and *in vivo* models of the emerging interest of naturally occurring and synthetic isothiocyanates as anticancer agents [51,52] highlight the interest of this chemical feature in novel small-molecule inhibitors of APE1.

Interestingly, although compounds 37 and 41 are less potent than compound 22, all the inhibitors have important sulfonate groups, similarly to the approved trypanocidal drug suramin which has been widely shown to have antitumour and antiangiogenic effects [53,54]. In fact, anticancer properties of suramin, a polysulfonated compound, have been revisited and a potential sensitising effect of cancer cells to IR mediated by inactivation of DNA-dependent protein kinase and impairment of DSB repair has been hypothesised [55]. Likewise, suramin has also been studied in combination with chemotherapeutic drugs to prevent the metastatic processes [56,57]. Sulfonate groups appear indeed to be essential for the establishment of hydrogen bonds and ionic interactions with APE1 residues to retain the inhibitors in the active site. Compounds 37 and 41 are also somehow structurally related to polysulfonated azo dyes some of

them with interesting *in vitro* cytotoxic properties [54,55,58,59]. In addition, two compounds containing sulfonate groups have been suggested as APE1 inhibitors by another research group [41]. However, compounds 22, 37 and 41 described in this thesis are considerably less bulky than the compounds previously reported being more prone to cross cellular membranes and to further optimisation to obtain APE1 inhibitors more potent and with more favourable pharmacokinetic and pharmacodynamic features. Moreover, these compounds did not reveal cytotoxic effects in MCF10A cells. Importantly, to best of our knowledge, compound 22 is the first APE1 inhibitor with both isothiocyanate and sulfonate groups in its structure, being a potential lead compound for development of novel APE1 inhibitors. Further optimisation of these compounds may include the introduction of chemical moieties that would force the formation of hydrogen bonds with the APE1 portion with no apparent interactions according with the docking analysis results. These structural modifications may include the addition of a short alkane chain bearing an H-bond acceptor group in the compounds core structure to promote the binding to residues Tyr128 and Tyr171 of APE1 active site. The absorption, distribution, metabolism and excretion (ADME) studies and quantitative structure-activity relationship (QSAR) analysis of these compounds may also result in more stable and potent APE1 endonuclease activity inhibitors.

The potential of compounds 22, 37 and 41 to sensitise the effects of chemotherapeutic drugs should be estimated in future studies to validate its development as APE1 inhibitors. Nevertheless, preclinical studies of novel compounds with promising therapeutic properties should be carefully designed to elucidate their action mechanism and properly estimate the relevance of proceed with their development. Iniparib, which was initially considered a potent PARP1 inhibitor, is an important example of the relevance of preclinical stage of drug discovery and development. The promising results attributed to iniparib in a multicenter, open-label, randomized phase II trial in combination with gemcitabine and carboplatin in patients with metastatic TNBC were not recapitulated in a phase III clinical trial [31,60]. The further assessment of *in vitro* ability of iniparib to inhibit PARP1 revealed that probably this compound has a different mechanism of action [61,62]. Therefore, the sensitising potential of compounds 22, 37 and 41 in terms of cytotoxic and genotoxic effects should be thoroughly evaluated in a panel of breast cancer cell lines with different degrees of aggressiveness and invasiveness due to possible cell type-specific effects. Since 3D cell culture models better mimic tumour microenvironment including cellular heterogeneity and hypoxic gradients, they should be also considered in this early phase of development [63]. Additionally, the complexity and redundancy of DNA repair pathways as well as the

plethora of lesions that can be induced by standard chemotherapeutic drugs require the selection of several anticancer agents to evaluate the potential of APE1 inhibitors to be used in combination therapy in breast cancer.

Therapeutic opportunities of targeting APE1 in breast cancer should not be restricted to the modulation of its DNA repair endonuclease activity. The emerging role of ROS and redox regulation as promising targets for anticancer drugs discovery emphasises the importance of elucidate putative anticancer effects of APE1 redox function modulation. In fact, APE1 redox-mediated activation of transcription factors renders this BER enzyme an interesting upstream effector of several signalling pathways essential for multiple cellular processes involved in cancer progression and development [64].

The well-known commercially available redox inhibitor of APE1 E3330 was selected to unveil the effects of the inhibition of this attractive function of the enzyme. Since taxanes act by disrupting microtubules dynamic to affect cytoskeleton function and APE1 was also shown to participate in the cell adhesion, remodelling of ECM, reorganization of actin cytoskeleton and microtubules structure, cell migration and invasion, DTX was also evaluated in combination with E3330 (Chapter 5). Theoretically, a therapeutic approach based on the combination of DTX with the APE1 redox inhibitor may lead to the impairment of migration and invasion of breast cancer cells and constitute a novel strategy in the treatment of metastatic disease. As far as we know, herein is reported the first study of this type of anticancer drugs combination in MDA-MB-231 cells.

Cellular migration is a complex event that requires several molecular and morphological modifications [65,66]. For breast cancer cells, single-cell and collective cell migration have been implicated in cancer metastases [65,66]. Therefore two mechanistically different migration assays namely wound healing and chemotaxis assay were chosen to mitigate the limitations of both methods in the evaluation of the combination of E3330 with DTX in the migration of MDA-MB-231 cells. E3330 alone markedly reduced collective cell migration in the wound healing assay whereas the combined treatment with DTX decreased chemoinvasion. Another important aspect of metastases development is the cellular invasion of surrounding tissues. In the chemoinvasion assay, the combination of E3330 with DTX significantly decreased the invasion of MDA-MB-231 cells. Future work should include the assessment of the expression of transcription factors under APE1 redox control and involved in cell migration and invasion (e.g. NF-ĸB, AP-1, STAT3, MMPs). The understanding of the action mechanism of the combination of E3330 with DTX at the molecular level as well

as the confirmation of these results with different concentrations of both drugs would be required to characterize the additive or synergistic effect of the drug combination and translate these findings to different cancer cell types. [67].

Overall, the multidisciplinary work herein presented integrated multiple endpoints being at the interface of intimately related disciplines essential for design and development of novel therapeutic approaches for cancer, namely Toxicology, Pharmacology and Chemistry. This innovative study included the evaluation of targeting different functions of APE1 with small-molecule inhibitors to improve the outcomes of conventional chemotherapeutic agents. Importantly, it further enabled the identification of three novel chemical entities suitable to be used as lead compounds to the development of more potent inhibitors of APE1 endonuclease function, placing APE1 as an attractive target in cancer field due to its unique properties as DNA repair enzyme and redox signalling factor. The assessment of similar endpoints in 3D cell models should be the next step to characterize the value of the described therapeutic strategy before considering the in vivo models. While important highlights about the role of APE1 as potential anticancer target were addressed, many questions in the vast field of DNA repair systems and surveillance mechanisms to maintain the integrity of genetic material remain to be uncovered.

REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J cancer. 2015;136:E359–E386.
2. Cardoso F, Harbeck N, Fallowfield L, Kyriakides S, Senkus E, ESMO Guidelines Working Group. Locally recurrent or metastatic breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2012;23 Suppl 7:vii11–vii19.
3. Cardoso F, Costa A, Norton L, Senkus E, Aapro M, André F, et al. ESO-ESMO 2nd international consensus guidelines for advanced breast cancer (ABC2). Ann Oncol. 2014;25:1871–1888.
4. Kakolyris S, Kaklamanis L, Engels K, Fox SB, Taylor M, Hickson ID, et al. Human AP endonuclease 1 (HAP1) protein expression in breast cancer correlates with lymph node status and angiogenesis. Br J Cancer. 1998;77:1169–1173.
5. Puglisi F, Barbone F, Tell G, Aprile G, Pertoldi B, Raiti C, et al. Prognostic role of Ape/Ref-1 subcellular expression in stage I-III breast carcinomas. Oncol Rep. 2002;9:11–17.
6. Woo J, Park H, Sung SH, Moon BI, Suh H, Lim W. Prognostic value of human apurinic/apyrimidinic endonuclease 1 (APE1) expression in breast cancer. PLoS One. Public Library of Science; 2014;9: e99528.
7. Chavez KJ, Garimella S V, Lipkowitz S. Triple negative breast cancer cell lines: one tool in the search for better treatment of triple negative breast cancer. Breast Dis.2010;32:35–48.
8. Holliday DL, Speirs V, Gey G, Coffman W, Kubicek M, Lasfargues E, et al. Choosing the right cell line for breast cancer research. Breast Cancer Res. 2011;13:215.
9. Lacroix M, Leclercq G. Relevance of breast cancer cell lines as models for breast tumours: an update. Breast Cancer Res Treat. 2004;83:249–289.
10. Sato S, Kopitz C, Grismayer B, Beaufort N, Reuning U, Schmitt M, et al. Overexpression of the urokinase receptor mRNA splice variant uPAR-del4/5 affects tumor-associated processes of breast cancer cells in vitro and in vivo. Breast Cancer Res Treat. 2011;127:649–657.
11. Tu YF, Kaipparettu BA, Ma Y, Wong LJC. Mitochondria of highly metastatic breast cancer cell line MDA-MB-231 exhibits increased autophagic properties. Biochim Biophys Acta. 2011;1807:1125–1132.
12. Fantozzi A, Christofori G, Weigelt B, Peterse J, Veer L van't, Fisher B, et al. Mouse models of breast cancer metastasis. Breast Cancer Res. 2006;8:212.
13. Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, et al. Genes that mediate breast cancer metastasis to lung. Nature. 2005;436:518–524.
14. Evans MJ, Saghatelian A, Sorensen EJ, Cravatt BF. Target discovery in small-molecule cell-based screens by in situ proteome reactivity profiling. Nat Biotechnol. 2005;23:1303–1307.

15. Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature. 2005;434:913–917.

16. Nowsheen S, Cooper T, Stanley JA, Yang ES. Synthetic lethal interactions between EGFR and PARP inhibition in human triple negative breast cancer cells. PLoS One. 2012;7:e46614.

17. Fernandes AS, Flórido A, Saraiva N, Cerqueira S, Ramalhete S, Cipriano M, et al. Role of the copper(II) complex Cu[15]pyN5 in intracellular ROS and breast cancer cell motility and invasion. Chem Biol Drug Des. 2015;86:578–588.

18. Zang C, Eucker J, Habbel P, Neumann C, Schulz CO, Bangemann N, et al. Targeting multiple tyrosine kinase receptors with Dovitinib blocks invasion and the interaction between tumor cells and cancer-associated fibroblasts in breast cancer. Cell Cycle. 2015;14:1291–1299.

19. Ribeiro IAC, Faustino CMC, Guerreiro PS, Frade RFM, Bronze MR, Castro MF, et al. Development of novel sophorolipids with improved cytotoxic activity toward MDA-MB-231 breast cancer cells. J Mol Recognit. 2015;28:155–165.

20. Bandarra S, Fernandes AS, Magro I, Guerreiro PS, Pingarilho M, Churchwell MI, et al. Mechanistic insights into the cytotoxicity and genotoxicity induced by glycidamide in human mammary cells. Mutagenesis. 2013;28:721–729.

21. Gonçalves S, Fernandes AS, Oliveira NG, Marques J, Costa J, Cabral MF, et al. Cytotoxic effects of cadmium in mammary epithelial cells: protective role of the macrocycle [15]pyN5. Food Chem Toxicol. 2012;50:2180–2187.

22. Tacar O, Sriamornsak P, Dass CR. Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems. J Pharm Pharmacol. 2013;65:157–170.

23. Gewirtz DA. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. Biochem Pharmacol. 1999;57:727–741.

24. Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol Rev. 2004;56:185–229.

25. Hernández-Vargas H, Palacios J, Moreno-Bueno G. Molecular profiling of docetaxel cytotoxicity in breast cancer cells: uncoupling of aberrant mitosis and apoptosis. Oncogene. 2007;26:2902–2913.

26. Alexandre J, Batteux F, Nicco C, Chéreau C, Laurent A, Guillevin L, et al. Accumulation of hydrogen peroxide is an early and crucial step for paclitaxel-induced cancer cell death both in vitro and in vivo. Int J cancer. 2006;119:41–48.

27. Rabi T, Bishayee A. d-Limonene sensitizes docetaxel-induced cytotoxicity in human prostate cancer cells: Generation of reactive oxygen species and induction of apoptosis. J Carcinog. 2009;8:9.

28. McGrogan BT, Gilmartin B, Carney DN, McCann A. Taxanes, microtubules and chemoresistant breast cancer. Biochim Biophys Acta. 2008;1785:96–132.

29. Franken NAP, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells in vitro. Nat Protoc. 2006;1:2315–2319.

30. Fenech M. Cytokinesis-block micronucleus cytome assay. Nat Protoc. 2007;2: 1084–1104.

31. O'Shaughnessy J, Schwartzberg L, Danso MA, Miller KD, Rugo HS, Neubauer M, et al. Phase III study of iniparib plus gemcitabine and carboplatin versus gemcitabine and carboplatin in patients with metastatic triple-negative breast cancer. J Clin Oncol. 2014;32:3840–3847.

32. Guha M. PARP inhibitors stumble in breast cancer. Nat Biotechnol. 2011;29: 373–374.

33. Hadi MZ, Wilson DM 3rd. Second human protein with homology to the Escherichia coli abasic endonuclease exonuclease III. Environ Mol Mutagen. 2000;36:312–324.

34. Burkovics P, Szukacsov V, Unk I, Haracska L. Human Ape2 protein has a 3'-5' exonuclease activity that acts preferentially on mismatched base pairs. Nucleic Acids Res. 2006;34:2508–2515.

35. Burkovics P, Hajdú I, Szukacsov V, Unk I, Haracska L. Role of PCNA-dependent stimulation of 3'-phosphodiesterase and 3'-5' exonuclease activities of human Ape2 in repair of oxidative DNA damage. Nucleic Acids Res. 2009;37:4247–4255.

36. Guikema JEJ, Gerstein RM, Linehan EK, Cloherty EK, Evan-Browning E, Tsuchimoto D, et al. Apurinic/apyrimidinic endonuclease 2 is necessary for normal B cell development and recovery of lymphoid progenitors after chemotherapeutic challenge. J Immunol. 2011;186:1943–1950.

37. Madhusudan S, Smart F, Shrimpton P, Parsons JL, Gardiner L, Houlbrook S, et al. Isolation of a small molecule inhibitor of DNA base excision repair. Nucleic Acids Res. 2005;33:4711–4724.

38. Bapat A, Glass LS, Luo M, Fishel ML, Reed A, Long EC, et al. Novel small-molecule inhibitor of apurinic/apyrimidinic endonuclease 1 blocks proliferation and reduces viability of glioblastoma cells. J Pharmacol Exp Ther. 2010;334:988–998.

39. Bapat A, Fishel ML, Kelley MR. Going ape as an approach to cancer therapeutics. Antioxid Redox Signal. 2009;11:651–668.

40. Manvilla BA, Pozharski E, Toth EA, Drohat AC. Structure of human apurinic/apyrimidinic endonuclease 1 with the essential Mg2+ cofactor. Acta Crystallogr D Biol Crystallogr. 2013;69:2555–2562.

41. Simeonov A, Kulkarni A, Dorjsuren D, Jadhav A, Shen M, McNeill DR, et al. Identification and characterization of inhibitors of human apurinic/apyrimidinic endonuclease APE1. PLoS One. 2009;4:e5740.

42. Zawahir Z, Dayam R, Deng J, Pereira C, Neamati N. Pharmacophore guided discovery of small-molecule human apurinic/apyrimidinic endonuclease 1 inhibitors. J Med Chem. 2009;52: 20–32.

43. Seiple LA, Cardellina JH, Akee R, Stivers JT. Potent inhibition of human apurinic/apyrimidinic endonuclease 1 by arylstibonic acids. Mol Pharmacol. 2008;73: 669–677.

44. Mohammed MZ, Vyjayanti VN, Laughton CA, Dekker L V, Fischer PM, Wilson DM 3rd, et al. Development and evaluation of human AP endonuclease inhibitors in melanoma and glioma cell lines. Br J Cancer. 2011;104:653–663.

45. Aiello F, Shabaik Y, Esqueda A, Sanchez TW, Grande F, Garofalo A, et al. Design and synthesis of 3-carbamoylbenzoic acid derivatives as inhibitors of human apurinic/apyrimidinic endonuclease 1 (APE1). ChemMedChem. 2012;7:1825–1839.

46. Ruiz FM, Francis SM, Tintoré M, Ferreira R, Gil-Redondo R, Morreale A, et al. Receptor-based virtual screening and biological characterization of human apurinic/apyrimidinic endonuclease (Ape1) inhibitors. ChemMedChem. 2012;7: 168–178.

47. Srinivasan A, Wang L, Cline CJ, Xie Z, Sobol RW, Xie XQ, et al. Identification and characterization of human apurinic/apyrimidinic endonuclease-1 inhibitors. Biochemistry. 2012;51:6246–6259.

48. Feng Z, Kochanek S, Close D, Wang L, Srinivasan A, Almehizia AA, et al. Design and activity of AP endonuclease-1 inhibitors. J Chem Biol. 2015;8:79–93.

49. Rai G, Vyjayanti VN, Dorjsuren D, Simeonov A, Jadhav A, Wilson DM 3rd, et al. Synthesis, biological evaluation, and structure–activity relationships of a novel class of apurinic/apyrimidinic endonuclease 1 inhibitors. J Med Chem. 2012;55:3101–3112.

50. Al-Safi RI, Odde S, Shabaik Y, Neamati N. Small-molecule inhibitors of APE1 DNA repair function: an overview. Curr Mol Pharmacol. 2012;5:14–35.

51. Jakubikova J, Cervi D, Ooi M, Kim K, Nahar S, Klippel S, et al. Anti-tumor activity and signaling events triggered by the isothiocyanates, sulforaphane and phenethyl isothiocyanate, in multiple myeloma. Haematologica. 2011;96:1170–1179.

52. Pham NA, Jacobberger JW, Schimmer AD, Cao P, Gronda M, Hedley DW. The dietary isothiocyanate sulforaphane targets pathways of apoptosis, cell cycle arrest, and oxidative stress in human pancreatic cancer cells and inhibits tumor growth in severe combined immunodeficient mice. Mol Cancer Ther. 2004;3:1239–1248.

53. Powis G, Seewald MJ, Melder D, Hoke M, Gratas C, Christensen TA, et al. Inhibition of growth factor binding, Ca2+ signaling and cell growth by polysulfonated azo dyes related to the antitumor agent suramin. Cancer Chemother Pharmacol. 1992;31:223–228.

54. Morris AD, Léonce S, Guilbaud N, Tucker GC, Pérez V, Jan M, et al. Eriochrome Black T, structurally related to suramin, inhibits angiogenesis and tumor growth in vivo. Anticancer Drugs. 1997;8:746–755.

55. Hosoi Y, Matsumoto Y, Tomita M, Enomoto A, Morita A, Sakai K, et al. Phosphorothioate oligonucleotides, suramin and heparin inhibit DNA-dependent protein kinase activity. Br J Cancer. 2002;86:1143–1149.

56. Borges S, Döppler HR, Storz P. A combination treatment with DNA methyltransferase inhibitors and suramin decreases invasiveness of breast cancer cells. Breast Cancer Res Treat. 2014;144:79–91.

57. Singla AK, Bondareva A, Jirik FR. Combined treatment with paclitaxel and suramin prevents the development of metastasis by inhibiting metastatic colonization of circulating tumor cells. Clin Exp Metastasis. 2014;31:705–714.

58. Brunner G, Zalkow L, Burgess E, Rifkin DB, Wilson EL, Gruszecka-Kowalik E, et al. Inhibition of glycosylphosphatidylinositol (GPI) phospholipase D by suramin-like compounds. Anticancer Res. 1996;16:2513–2516.

59. Langer I, Atassi G, Robberecht P, Résibois A. Eriochrome Black T inhibits endothelial cell growth through S-phase blockade. Eur J Pharmacol. 2000;399:85–90.

60. O'Shaughnessy J, Osborne C, Pippen JE, Yoffe M, Patt D, Rocha C, et al. Iniparib plus chemotherapy in metastatic triple-negative breast cancer. N Engl J Med. 2011;364:205–214.

61. Patel AG, De Lorenzo SB, Flatten KS, Poirier GG, Kaufmann SH. Failure of iniparib to inhibit poly(ADP-Ribose) polymerase in vitro. Clin Cancer Res. 2012;18: 1655–1662.

62. Liu X, Shi Y, Maag DX, Palma JP, Patterson MJ, Ellis PA, et al. Iniparib nonselectively modifies cysteine-containing proteins in tumor cells and is not a bona fide PARP inhibitor. Clin Cancer Res. 2012;18:510–523.

63. Thoma CR, Zimmermann M, Agarkova I, Kelm JM, Krek W. 3D cell culture systems modeling tumor growth determinants in cancer target discovery. Adv Drug Deliv Rev. 2014;69-70:29–41.

64. Wondrak GT. Redox-directed cancer therapeutics: molecular mechanisms and opportunities. Antioxid Redox Signal. 2009;11:3013–3069.

65. Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. Nat Rev Cancer. 2003;3:362–374.

66. Yilmaz M, Christofori G. Mechanisms of motility in metastasizing cells. Mol Cancer Res. 2010;8:629–642.

67. Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res. 2010;70: 440–446.

ANNEX

This annex refers to supporting information of Chapter 4 which was adapted from:

Guerreiro PS, Estácio SG, Antunes F, Fernandes AS, Pinheiro PF, Costa JG, Castro M, Miranda JP, Guedes RC, Oliveira NG. Structure-based virtual screening toward the discovery of novel inhibitors of the DNA repair activity of the human apurinic/apyrimidinic endonuclease 1. Chem Biol Drug Des. 2016;88:915-925.

INDEX

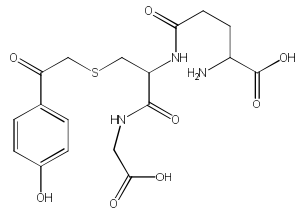
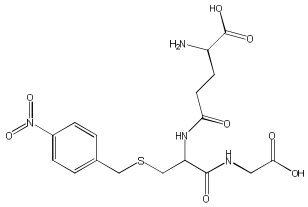
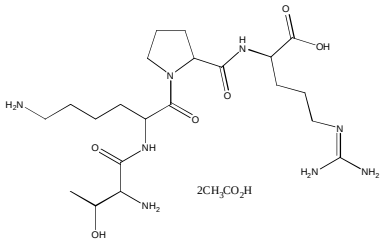
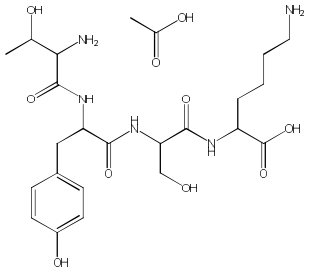
EXPERIMENTAL DETAILS FOR STRUCTURE CONFIRMATION AND PURITY ASSESSMENT

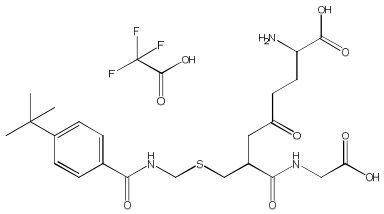
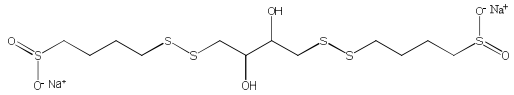
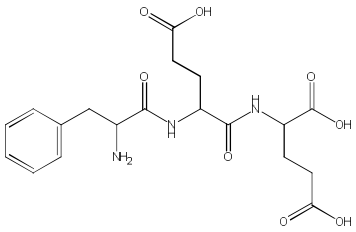
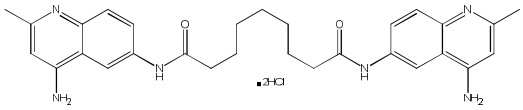
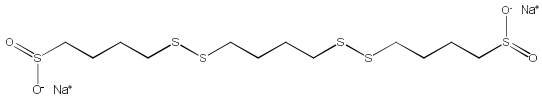
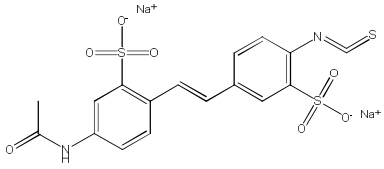
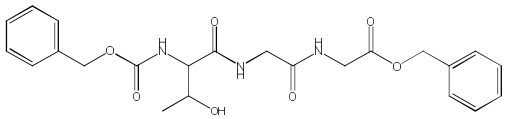
Purity of each compound was determined by HPLC-DAD on a Ultimate 3000 Dionex system, consisting of an LPG-3400A quaternary gradient pump and a diode array spectrophotometric detector (Dionex Co., Sunnyvale, California, USA), and equipped with a Rheodyne model 8125 injector (Rheodyne, Rohnert Park, California, USA). HPLC analyses were performed with a Luna C18 (2) column (250 mm × 4.6 mm; 5 µm; Phenomenex, Torrance, California, USA), at a flow rate of 1 mL.min−1. A 30 min linear gradient from 5 to 70% acetonitrile in 0.1% aqueous formic acid, followed by a 2 min linear gradient to 100% acetonitrile and an 8 min isocratic elution with acetonitrile, was used in all instances. The UV absorbance was monitored at 254 nm.

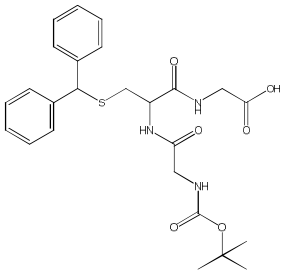
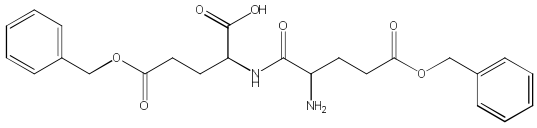
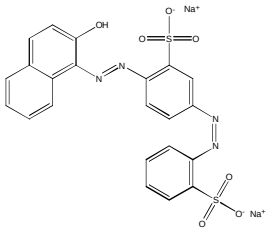
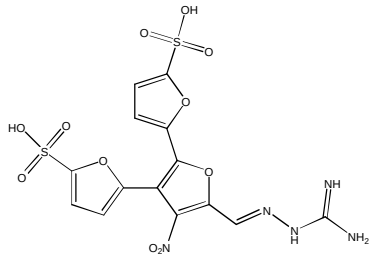
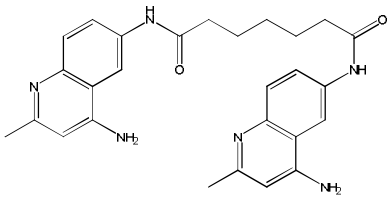
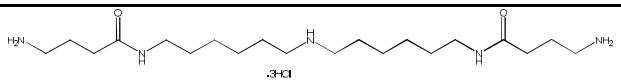
Chemical identity was confirmed for all compounds by high-resolution mass spectrometry analysis on a Impact II ESI-Qq-TOF mass spectrometer (Bruker Daltoniks, Bremen, Germany). Mass spectra were obtained with the ESI interface operating in negative ion mode. The optimized ESI parameters were: ion spray voltage, 4.5 kV; drying gas (nitrogen) temperature, 200 ºC; drying gas flow, 4 L/min; nebulising gas (nitrogen) pressure, 0.3 bar. The detection was performed considering a mass range of 100-2000 m/z. The accurate mass data of the molecular ions were processed through the software DataAnalysis 4.1 (Bruker Daltoniks).

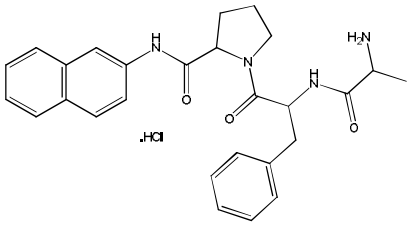
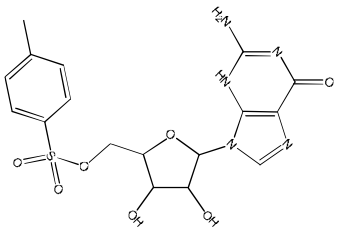
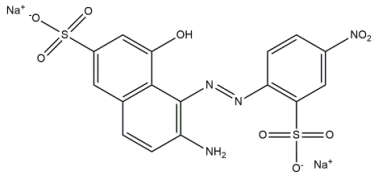
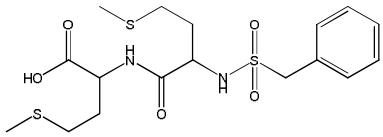
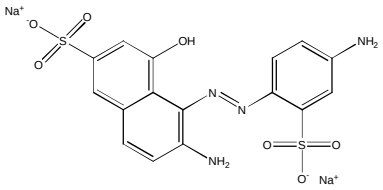
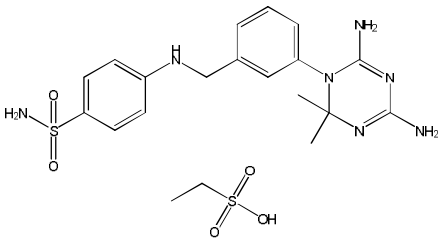
STRUCTURE OF COMPOUNDS TESTED IN APE1 ENDONUCLEASE ACTIVITY ASSAY

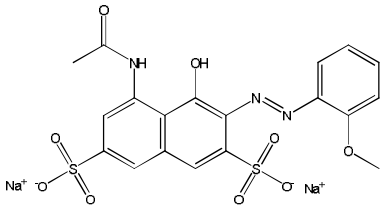
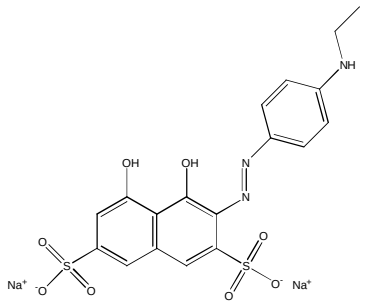
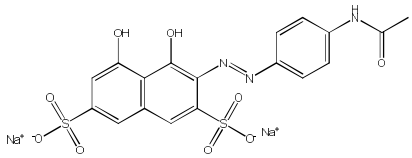
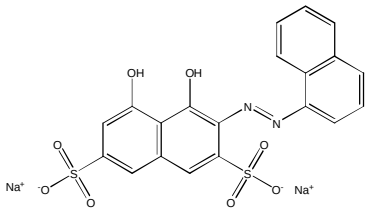
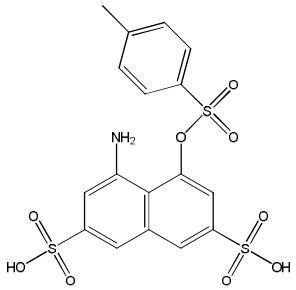
Table S1 Structure of the 30 compounds identified in the *in silico* study and selected to be tested in the first screen using the fluorescence-based APE1 endonuclease activity assay. Each compound was evaluated at the final concentration of 50 µM and the results of APE1 endonuclease activity are presented relatively to the negative control.

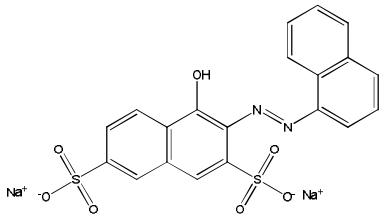
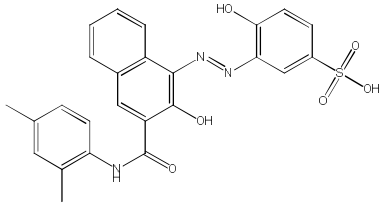
Structure	NSC number	APE1 endonuclease activity (% of control)
 **Compound 2**	NSC 131129	>100
 **Compound 4**	NSC 131122	>100
 **Compound 6**	NSC 341953	>100
 **Compound 7**	NSC 344833	>100

 **Compound 8**	NSC 602629	>100
 **Compound 13**	NSC 624203	91.4[a)]
 **Compound 14**	NSC 343026	89.1 ± 13.1
 **Compound 16**	NSC 22908	5.8 ± 4.8
 **Compound 18**	NSC 624205	>100
 **Compound 22**	NSC 378144	0
 **Compound 24**	NSC 343730	>100

 **Compound 25**	NSC 338500	>100
 **Compound 27**	NSC 668891	99.6 ± 1.6
 **Compound 28**	NSC 10469	50.6 ± 4.0
 **Compound 29**	NSC 647123	>100
 **Compound 32**	NSC 22904	>100
 **Compound 33**	NSC 109232	>100

 **Compound 34**	NSC 339934	>100
 **Compound 36**	NSC 62628	>100
 **Compound 37**	NSC 47703	10.9 ± 0.3
 **Compound 38**	NSC 401341	>100
 **Compound 41**	NSC 401610	4.7 ± 0.4
 **Compound 47**	NSC 368892	>100

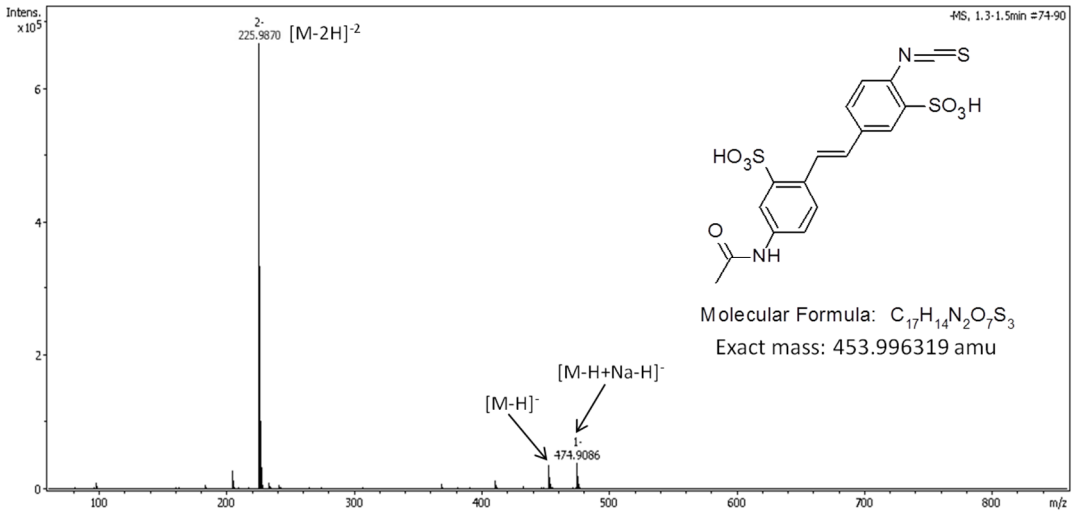
 **Compound 48**	NSC 47711	17.3 ± 3.1
 **Compound 49**	NSC 16209	12.4 ± 0.1
 **Compound 50**	NSC 45201	39.9 ± 10.7
 **Compound 51**	NSC 45208	26.2 ± 1.9
 **Compound 52**	NSC 16168	16.3 ± 2.5

 **Compound 53**	NSC 45207	72.5 ± 6.3
 **Compound 54**	NSC 97318	23.1 ± 2.6

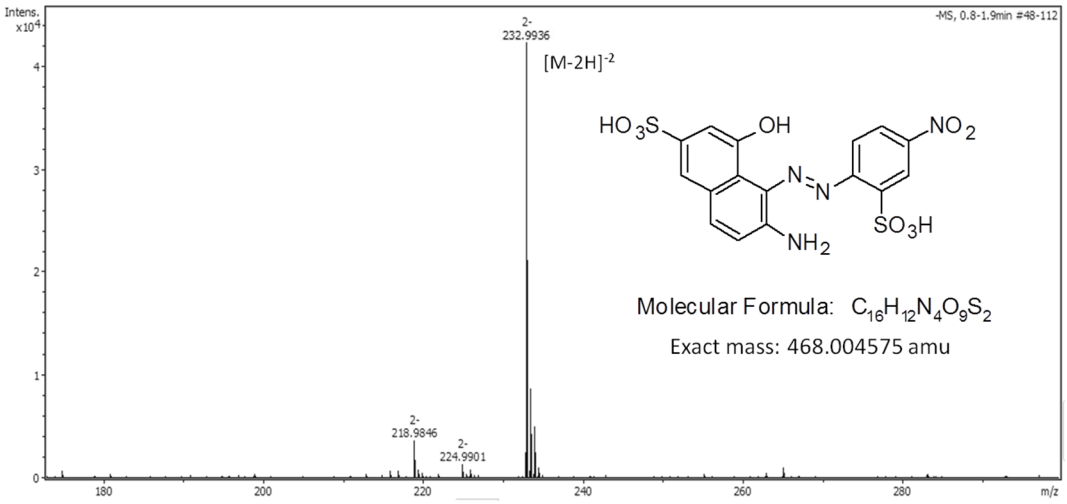
a) Only one experiment performed with two replicates was considered.

MASS SPECTRA FOR ACTIVE COMPOUNDS

Compound 22



Compound 37



Compound 41

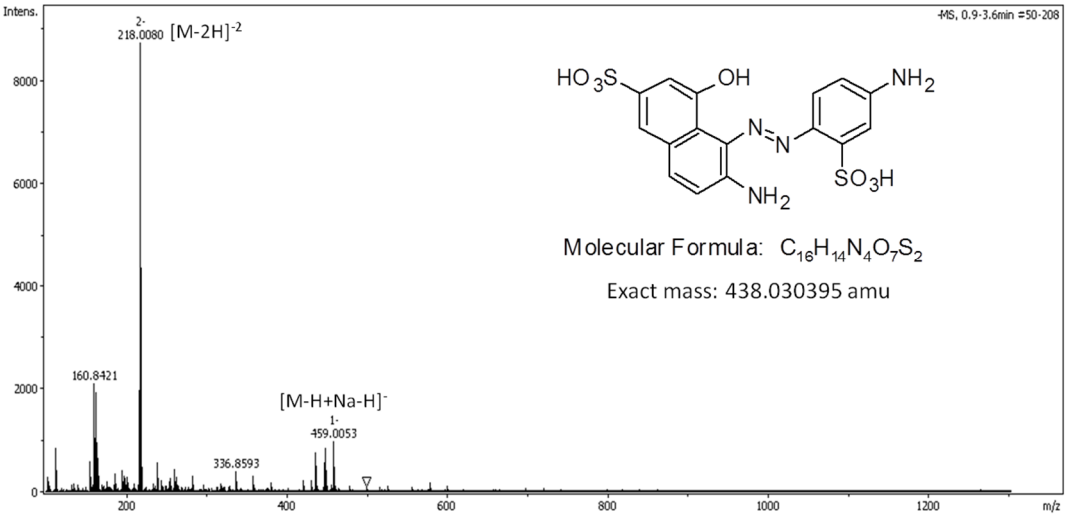


Figure S1 Mass spectra (ESI-) of compounds 22, 37 and 41.

DETERMINATION OF THE MODE OF ENZYMATIC INHIBITION OF COMPOUND 22

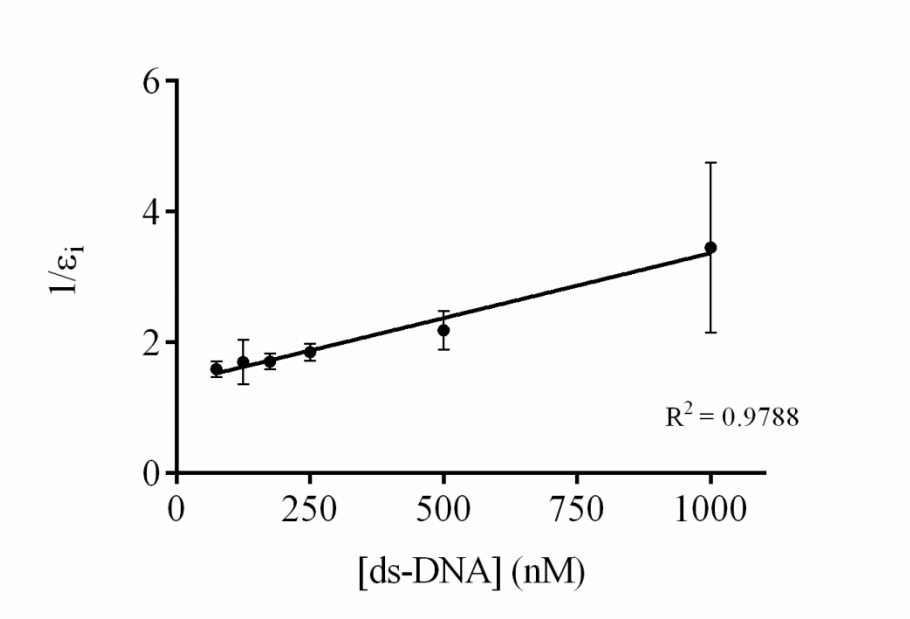


Figure S2 Determination of the mode of enzymatic inhibition of compound 22. Compound 22 (250 nM) seems to present a competitive mechanism of APE1 inhibition as evaluated by the plot of $1/\varepsilon_i$ (where ε_i is the degree of inhibition) versus substrate (ds-DNA) concentration. Values represent the mean ± SD (n = 3).